

Historic, Archive Document

Do not assume content reflects current scientific knowledge, policies, or practices.



Aflatoxin Handbook

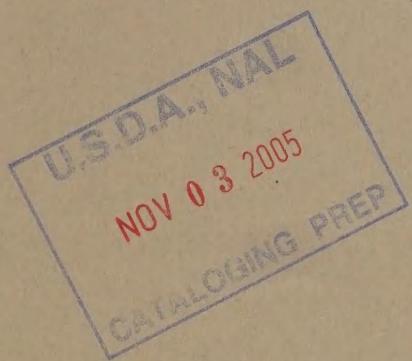
**United States
Department of
Agriculture**

Grain Inspection,
Packers and
Stockyards
Administration

Federal Grain
Inspection
Service

Washington, DC

March 2002



United States Department of Agriculture
Grain Inspection, Packers and Stockyards Administration
Packers and Stockyards Administration

Program Handbook

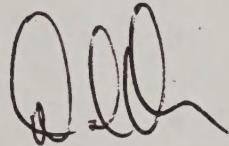
3-4-02

Aflatoxin Testing

Foreword

The Aflatoxin Handbook has been revised to incorporate changes to the aflatoxin testing program that have evolved since the handbook was last printed on 2-21-92.

This handbook illustrates step-by-step procedures for testing and certifying grain and commodities for aflatoxin. All official inspection personnel authorized or licensed to perform aflatoxin testing shall reference this handbook for procedures.



David Orr, Director
Field Management Division

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

AFLATOXIN HANDBOOK
TABLE OF CONTENTS
3-4-02

Aflatoxin Testing

TABLE OF CONTENTS

CHAPTER	TITLE
1	GENERAL INFORMATION
2	LABORATORY SAFETY
3	SAMPLE PREPARATION
4	CERTIFICATION
5	AFLACUP TEST KIT
6	EZ-SCREEN TEST KIT
7	AGRI-SCREEN TEST KIT
8	AFLATEST TEST METHOD
9	FLUOROQUANT TEST METHOD
10	VERATOX-AST TEST KIT

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, DC 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 1
3-4-02

CHAPTER 1

GENERAL INFORMATION

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
1.1	PURPOSE	1-1
1.2	BACKGROUND	1-1
1.3	MANDATORY TESTING	1-2
1.4	CONTAMINATION LIMITS	1-2
1.5	APPROVED TEST METHODS	1-3
1.6	DISCLAIMER CLAUSE	1-5
1.7	TESTING SERVICES	1-5
1.8	REVIEW INSPECTIONS	1-6
1.9	QUALITY ASSURANCE PROGRAM	1-8

ATTACHMENT: AFLATOXIN SAMPLING AND RECONDITIONING PROCEDURES

1.1 PURPOSE

This handbook establishes official procedures for determining aflatoxin in grain and processed grain products, and certifying the official results.

1.2 BACKGROUND

Aflatoxin is a naturally occurring mycotoxin produced by two types of mold: *Aspergillus flavus* and *Aspergillus parasiticus*. *Aspergillus flavus* is common and widespread in nature and is most often found when certain grains are grown under stressful conditions such as drought. The mold occurs in soil, decaying vegetation, hay, and grains undergoing microbiological deterioration and invades all types of organic substrates whenever and wherever the conditions are favorable for its growth. Favorable conditions include high moisture content and high temperature. At least 13 different types of aflatoxin are produced in nature with aflatoxin B1 considered as the most toxic. While the presence of *Aspergillus flavus* does not always indicate harmful levels of aflatoxin it does mean that the potential for aflatoxin production is present.

GIPSA provides aflatoxin testing service as official criteria for corn, sorghum, wheat, and soybeans, as official criteria under the United States Grain Standards Act (USGSA). Testing is also provided for rice, popcorn, corn meal, corn gluten meal, corn/soy blend, and other processed products governed by the Agricultural Marketing Act (AMA).

Aflatoxin testing services are available nationwide, upon request and for a fee, as either a qualitative (screening above or below a threshold determined by the customer) or as a quantitative (actual results in parts per billion) service using several different types of test kits approved by GIPSA.

To further assist the grain industry, GIPSA also provides, on a limited basis, a complex chemical testing method, High Performance Liquid Chromatography (HPLC) testing for aflatoxin. The HPLC testing procedure is performed, upon request, for Board Appeal inspections only. All official aflatoxin testing is performed as prescribed in the GIPSA directive by authorized employees of GIPSA or licensed delegated/designated agency personnel.

Individuals wanting official aflatoxin testing should contact the nearest FGIS field office or delegated/designated agency.

1.3 MANDATORY TESTING

The 1990 Farm Bill (Food, Agriculture, Conservation, and Trade Act of 1990, P.L. 101-624) amended section 5 of the USGSA to "... require that all corn exported from the United States be tested to ascertain whether it exceeds acceptable level of aflatoxin contamination, unless the contract for export between the buyer and seller stipulates that aflatoxin testing shall not be conducted."

1.4 CONTAMINATION LIMITS

The Food and Drug Administration (FDA) has established action levels for aflatoxin present in food or feed. These limits are established by the Agency to provide an adequate margin of safety to protect human and animal health.

FGIS and FDA, having certain related objectives in carrying out their respective regulatory and service functions, have an agreement (Memorandum of Understanding) to assure the most effective possible system for identifying lots of grain, rice, pulses, and food products which exceed the FDA action levels of aflatoxin contamination. Under the provisions of the Memorandum of Understanding (MOU), FGIS and officially delegated/designated agencies report to FDA, on a lot-by-lot basis, each lot (grain, rice, and processed products) that, during the course of an official sample-lot inspection, exceed the 20 ppb FDA action limit.

Listed below are the FDA action levels for aflatoxins in animal feeds.

20 ppb	For corn and other grains intended for immature animals (including immature poultry) and for dairy animals, or when its destination is not known;
20 ppb	For animal feeds, other than corn or cottonseed meal;
100 ppb	For corn and other grains intended for breeding beef cattle, breeding swine, or mature poultry;
200 ppb	For corn and other grains intended for finishing swine of 100 pounds or greater;
300 ppb	For corn and other grains intended for finishing (i.e., feedlot) beef cattle and for cottonseed meal intended for beef cattle, swine or poultry.

Aflatoxin-contaminated corn lots may be reconditioned under the certain conditions established by FDA. (See Attachment)

1.5 APPROVED TEST METHODS

FGIS has approved test kits for use at field testing locations. The AflaCup, EZ-Screen, and Agri-Screen test kits are approved for qualitative analysis of corn. The Aflatest, Fluoroquant, and Veratox-AST test kits provide quantitative analysis but can be used for qualitative results. HPLC testing is reserved for quantitative testing at TSD only.

The methods listed below have been conformance tested to perform within FGS specifications. Each of the approved test methods has been certified to provide results accurate up to the conformance test level at which they were approved.

FGIS APPROVED TEST METHODS			
Method and Test Kit	Approved for		Conformance Limit(s)
	Qualitative	Quantitative	
AflaCup (International Diagnostics Inc.)	X		20 ppb
EZ-Screen - (Editek, Inc.)	X		20 ppb
AgriScreen - (Neogen)	X		20 ppb
Veratox AST - (Neogen)	X	X	300 ppb (quantitative)
Fluoroquant - (Romer)	X	X	300 ppb (quantitative)
Aflatest	X	X	300 ppb (quantitative)

Listed in the table below are the test kits that are commonly used for official aflatoxin analysis. Use the table to determine the appropriate test kit(s) to use for testing the listed grain/commodity. For information concerning the testing of mixed grain, contact the Policies and Procedures Branch.

GRAIN/ COMMODITY	TEST METHOD					
	AflaCup	Aflatest	EZ-Screen	Agri-Screen	Fluoroquant	Veratox- AST
Corn	X	X	X	X	X	X
Sorghum		X			X	X
Wheat		X			X	X
Soybeans		X			X	X
Corn Screenings		(*)				(*)
Corn Meal		X			X	X
Corn Germ Meal		X				X
Corn Gluten Meal		X				X
Corn/Soy Blend		X			X	X
Corn Gluten Feed		X				
Flaking Corn Grits		X			(*)	(*)
Corn Flour						(*)
Corn Bran						(*)
Popcorn		X			X	X
Milled Rice		X			X	X
Rough Rice						(*)

NOTE: An X entered into a block denotes that the test kit has been evaluated and approved for the grain/commodity.

The symbol (*) entered into a block denotes that the test kit is under evaluation by the Technical Services Division (TSD) for the grain/commodity and is temporarily approved for official use.

1.6 DISCLAIMER CLAUSE

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

1.7 TESTING SERVICES

Applicants requesting aflatoxin testing must specify whether qualitative or quantitative testing service is desired. If qualitative analysis is requested, the applicant must specify the level desired (e.g., 20 ppb). Three types of aflatoxin testing services are available as follows:

a. Submitted Sample Service.

Analysis based on a sample submitted by the applicant for service.

b. Official Sample-Lot Service.

Analysis based on an official sample obtained and analyzed by official personnel.

(1) Single lot inspection.

Samples may be obtained and tested on either an individual carrier basis or a composite sample basis (maximum of five railcars or fifteen trucks per composite sample).

(2) Unit train inspection under the CuSum Loading Plan.

Unit trains are analyzed on a subplot basis for corn and sorghum and on a composite basis for other grains. Acceptable sublots must conform to contract specifications when "maximum" limits are specified.

When aflatoxin testing is required, samples may be obtained and tested on either an individual carrier basis or a subplot basis. The maximum size subplot for aflatoxin testing is five railcars for unit trains consisting of less than 200,000 bushels, or less than fifty railcars. For unit trains consisting of 200,000 bushels or more, or fifty railcars or more, the maximum subplot size is ten railcars.

(3) Export shiplots

Export shiplots are analyzed on a subplot basis for corn and sorghum and on a composite basis for other grains. Acceptable sublots must conform to contract specifications when "maximum" limits are specified.

(4) Supplemental Testing.

Upon request, supplemental testing may be performed as follows:

Composite samples may be analyzed in addition to the subplot test for corn and sorghum shiplots or unit trains.

(5) Alternate Testing.

Upon request, alternate testing methods may be used, provided that the minimum testing requirements are met. Examples of alternate testing are as follows:

- (a) Sublot testing may be used instead of composite sample analysis for grains routinely tested on a composite basis.
- (b) Grain shipments may be tested on a component sample basis in lieu of the subplot basis under the provisions of Book III, Inspection Procedures. Components are combined and averaged to determine the subplot result. Acceptable quality will be based on the subplot result as compared to the contracted "maximum" specification.

c. Warehouse Sample-Lot Inspection Service.

Analysis based on an official sample obtained by a licensed warehouse sampler and analyzed by official personnel.

1.8 REVIEW INSPECTIONS

Sections 800.125 and 800.135 of the USGSA permit a review inspection on either official grade/factors or official criteria. When requested, a review inspection for official grade or official factor and official criteria may be handled separately even though both sets of results are reported on the same certificate.

Review inspection services for aflatoxin are provided on either a new sample or the file sample in accordance with the regulations. Board appeal inspection services are limited to the analysis of file samples.

Only one field review (reinspection or appeal inspection) is permitted for shiplot, unit train, or lash barge material portions when testing is performed on a subplot basis. However, if the applicant requests a review of the entire lot, up to three review levels of service (reinspection, appeal, board appeal) may be obtained for each subplot included in the lot. Inspection results for each review level shall replace the previous inspection result.

a. Reinspection Service.

The laboratory providing original testing services also provides reinspection services. Applicants may request either qualitative or quantitative analysis unless the original test was quantitative. Then, only a quantitative analysis is available.

b. Appeal Inspection Service.

FGIS field offices provide appeal testing services for aflatoxin. Field offices not equipped to provide testing will make arrangements with another FGIS office to provide the most timely service possible. Applicants may request either qualitative or quantitative analysis unless the original or reinspection tests were quantitative. Then, only a quantitative analysis is available. If samples are sent to a field office for analysis, write the words "**AFLATOXIN APPEAL**" in the "Remarks" section of the grain sample ticket and on the back of the mailing tag.

c. Board Appeal Inspection Services.

Board appeal inspection services are limited to the file sample and are provided by the Board of Appeals and Review (BAR) in Kansas City. Applicants may request either qualitative or quantitative analysis unless the original or reinspection tests were quantitative. Then, only a quantitative analysis is available. The HPLC method is also available for determining aflatoxin in Board appeal samples. The applicant must specify the HPLC method as the desired determination method. Otherwise, the Board appeal inspection will be conducted using the rapid method (test kits).

When sending samples to the BAR, write the words "**AFLATOXIN BOARD APPEAL**" in the "Remarks" section of the grain sample ticket and on the back of the mailing tag.

1.9 QUALITY ASSURANCE PROGRAM

The Technical Services Division (TSD), located at the Kansas City Technical Center, conducts an aflatoxin check sample program for all specified service points and laboratories providing testing services. TSD is responsible for preparing and distributing check samples each quarter to all official aflatoxin testing locations, analyzing check sample results, notifying field locations of any results indicating problems, and releasing a quarterly summary report to all participating laboratories. Field offices are responsible for routine supervision to assure all laboratories in their circuit provide accurate results. The TSD check sample program is designed to test the capability of the official system and to monitor the accuracy of approved testing methods. The check sample program provides limited performance information that can be used to supplement the routine supervision of official personnel performing testing services.

AFLATOXIN SAMPLING AND RECONDITIONING PROCEDURES

1. FDA RECONDITIONING GUIDELINES

The Food and Drug Administration (FDA) will permit reconditioning of aflatoxin-contaminated corn lots at export locations by mechanical cleaning under the following conditions:

- a. Only one attempt at reconditioning is allowed. The analytical results from the reconditioned lot will be the final determination for disposition of the entire lot.
- b. To assure proper reconditioning, the grain company must mechanically clean the lot at a rate not to exceed 50 percent of the rated cleaner capacity.
- c. FGIS must oversee the cleaning process, sample the reconditioned lot (cleaned corn) using a diverter-type mechanical sampler, and analyze the samples for aflatoxin.
- d. FGIS must sample the cleanings/screenings using the most practical procedures available and test the cleanings and/or screenings for aflatoxin contamination.

At interior locations, the local FDA office may modify the reconditioning procedures to provide for a cost effective process.

2. FGIS RESPONSIBILITIES

When positive lots are identified at export locations, field office managers (FOM) should work with the grain facility representatives and develop a standard operating procedure (SOP) for reconditioning aflatoxin-contaminated corn.

FOM's should review the SOP with local FDA officials before implementing the reconditioning process, unless instructed otherwise by FDA.

- a. Export Locations

At export locations, FGIS or official delegated state agency personnel, as applicable, are responsible for:

- (1) Reporting actionable lots to the local FDA field office.
- (2) Preserving the identity of actionable lots prior to reconditioning.
- (3) Monitoring the reconditioning process at the grain facility.
- (4) Sampling and testing reconditioned lots (cleaned corn and screenings) for aflatoxin. When sampling screenings, use the most practical method available to obtain a representative sample.
- (5) Preserving the identity of reconditioned lots and screenings. (Screenings are not considered a reconditioned lot.)
- (6) Reporting aflatoxin results of reconditioned lots and screenings to FDA.
- (7) Completing a report of the reconditioning process. Include in the report the following information:
 - (a) Date Reconditioned.
 - (b) Grain Elevator/Location.
 - (c) Type of Sample/Carrier.
 - (d) Original Results.
 - (e) Reconditioned Whole Grain Results.
 - (f) Cleanings/Screenings Results.
 - (g) Size of Cleaner Screens used to Recondition the Lot.
 - (h) Elevator Set-up Information.

b. Domestic Locations

FOM's servicing interior locations should contact the local FDA office servicing the area where the contaminated lot is located to discuss and determine responsibilities for managing the reconditioning process. Official agencies and affected grain companies are encouraged to participate in these discussions to facilitate the development of an SOP.

AFLATOXIN HANDBOOK
CHAPTER 1
ATTACHMENT
3-4-02

3. SAMPLE SIZE AND PREPARATION

Obtain the minimum sample size as directed in chapter 2 of this handbook. If requested by the applicant, a larger sample size may be obtained.

Grind the entire corn sample obtained for aflatoxin testing and prepare three 500-gram subportions from the ground sample.

<u>Sample Portion</u>	<u>Use</u>
Test Portion	Original inspection service
File Portion	Review inspection service
FDA Portion	Retain for FDA analysis if results exceed 20 ppb.

When reconditioned lots are resampled in accordance with the FDA guidelines, a file portion is not required.

If FGIS's original results for a reconditioned lot of corn or screenings exceed 20 ppb, the FDA sample portion will be used for any subsequent verification (by FDA) of results.

4. DISPOSITION POLICY

The grain industry must comply with FDA policy regarding the disposition of corn and screenings resulting from the reconditioning process. In general, disposition will occur as follows:

- Cleanings/screenings may be used for animal feed if the aflatoxin content meets FDA feed guidelines. The screenings may not re-enter food channels in any fashion.
- Reconditioned (cleaned) corn with less than 20 ppb aflatoxin may be handled without restrictions. When the reconditioning process fails and the corn continues to exceed the 20 ppb level, disposition is based on current FDA policy.

Contact the local FDA office regarding other questions concerning specific disposition action.

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 2
3-4-02

CHAPTER 2

LABORATORY SAFETY

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
2.1	GENERAL INFORMATION.....	2-1
2.2	APPROVED FGIS LABORATORY SPACE	2-1
2.3	FGIS LABORATORY PRACTICES	2-3
2.4	STORING CHEMICALS AND SOLVENTS	2-4
2.5	CLEANING LABWARE	2-4
2.6	CLEANING AFLATOXIN SOLUTION SPILLS	2-5
2.7	DISPOSING EXCESS SAMPLE EXTRACT	2-6
2.8	WASTE DISPOSAL	2-6
2.9	FIELD OFFICE MANAGER RESPONSIBILITY	2-7

2.1 GENERAL INFORMATION

Aflatoxin testing requires the use of flammable liquids and suspected carcinogens. The building owner (private or GSA) must permit the use of methanol in space used by FGIS. FGIS will provide testing services onsite only in facilities that provide adequate protection to FGIS personnel. The following space requirements apply to FGIS occupied space only.

2.2 APPROVED FGIS LABORATORY SPACE

Individual elevators may provide two kinds of space for personnel to perform onsite aflatoxin testing. The space may be located (1) in a building along with other occupants or (2) in a building devoted exclusively to laboratory space.

In either case, the plan for the intended laboratory space is subject to inspection and approval by FGIS prior to construction. The Safety and Health Office, Equipment Branch, and field office manager will review proposed plans and suggest ways to comply with the requirements.

The following are minimum requirements for laboratory space:

a. Location.

Locate the laboratory at least 100 feet from the base of the elevator headhouse. This distance is subject to negotiation when the elevator uses exterior grain legs and/or inclined belts in lieu of interior grain legs or where the headhouse is equipped with blow-out panels or the headhouse consists of a lightly covered framework.

Laboratories must meet the following requirements when they are located in a building with other occupants.

- (1) Isolate the laboratory from nonlaboratory occupants using a fire barrier having at least a 1-hour fire resistance.
- (2) Provide a fire barrier consisting of floors, ceilings, and interior walls.
- (3) Provide all passageways and other openings that lead to adjacent interior space with self-closing fire doors having a 1-hour fire resistance. Do not block these doors open.

- (4) Separate the space from central heating, ventilation, and air-conditioning using automatic-closing fire dampers in the heating, ventilation, and air-conditioning ducts near the fire barrier, or provide a separate heating, ventilation, and air-conditioning system for the laboratory.

b. Size.

Dedicate the space strictly for laboratory (chemical) work. Supply adequate space for chemical analysis (minimum of 100 square feet) and a separate area for sample preparation and grinding purposes. Samples must be ground in space separate from the analytical space.

c. Electrical System.

Provide the laboratory space with electrical power and lighting meeting the standards of the National Electrical Code. Wiring suitable for a Class I location is not required. A three-wire system consisting of an energized wire, a neutral wire, and a grounding conductor is satisfactory.

Install overhead lighting fixtures through ceilings that serve as fire barriers. Fixtures suspended below such ceilings are acceptable.

d. Exhaust System.

The exhaust system must remove methanol vapors from the work area. Normal air conditioning and heating may provide adequate ventilation when performing testing procedures in a building devoted exclusively for laboratory space. The local Collateral Duty Safety and Health Officer and the Safety and Health Office in Washington, DC, will assist in assessing on a case-by-case basis whether added ventilation, such as a fume hood, is needed. If needed, situate the laboratory space so that hoods, to be supplied by FGIS, are vented to the exterior of the building. Fume hood ventilation will require a 6 or 8 inch diameter opening either vertically through the ceiling and roof or horizontally through an exterior wall. In some cases, a portable hood may be sufficient.

e. Plumbing.

Provide the laboratory space with a basin having hot and cold potable water and a sewer connection.

For further information about these requirements, contact the FGIS Safety and Health Staff.

f. Eyewash and Safety Shower Station.

Provide the laboratory space with eyewash equipment (eyewash bottle or permanent faucet-mounted fixture). A permanent, faucet-mounted eyewash fixture is highly recommended. A safety shower station must be installed in laboratories where acetonitrile-based extraction solvent (Romer-Fluoroquant test method) is used.

2.3 FGIS LABORATORY PRACTICES

When working in a laboratory, FGIS employees must comply with the Chemical Hygiene Plan developed for the laboratory where the testing is performed. To accomplish this, include the following as part of an overall FGIS laboratory "Standard Operating Procedure" (SOP). Maintain the SOP, this handbook, and the current Material Safety Data Sheets (MSDS) and Chemical Hygiene Plan at each laboratory.

- a. Label all bottles and containers according to the Hazard Communication Program. In addition, when preparing mixtures of solutions, securely apply a label with the name of the solution, the preparation date, and the preparer's initials written in permanent ink.
- b. Do not smoke, eat, drink, or chew gum or tobacco in the laboratory.
- c. Wash hands immediately before and after eating, drinking, and smoking outside of the laboratory area.
- d. Wear a disposable, fire-retardant laboratory coat and disposable impermeable gloves when working.
- e. Clean the laboratory equipment and dispose of contaminated materials according to procedures listed in this chapter.
- f. Wear an FGIS-approved disposable mask and hair protection when grinding samples, or when otherwise exposed to airborne grain dust.
- g. Do not wear contact lenses in the laboratory if the testing process involves chemicals other than methanol (e.g., acetonitrile).

- h. Wear FGIS-approved safety glasses or splash goggles when in the lab (also applies to visitors in the lab).
- i. Do not store food or drink in the laboratory refrigerator. Store only the test kits and other items requiring refrigeration.
- j. Do not wear protective clothing outside the laboratory unless waste chemicals are being removed to outside storage facilities or extra chemicals are being carried into the laboratory from an outside storage cabinet.
- k. Do not store masks and hair protectors in the grinding area where they might become contaminated by the dust particles.

2.4 STORING CHEMICALS AND SOLVENTS

- a. Store chemicals and equipment outside the fume hood.
- b. Store chemicals in places where they will not clutter bench tops or obstruct movements. Do not store solutions at a height exceeding eye level. Large bottles shall be stored no more than two feet above ground level.
- c. Prepare all solutions and perform analyses in a working fume hood.
- d. Limit the total quantity of waste chemicals in the laboratory to 1 liquid gallon.
- e. Maintain a current MSDS for each chemical at the laboratory. If each supply of chemicals received does not have a MSDS enclosed, contact the company and request one immediately.
- f. Limit the total amount of flammable solvent in the laboratory to 2 gallons.
- g. Store flammable solvents in an approved solvent storage cabinet.

2.5 CLEANING LABWARE

- a. Negative Tests (\leq 20 ppb).

- (1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

2.6 CLEANING AFLATOXIN SOLUTION SPILLS

Perform the following procedures only while wearing disposable impermeable gloves and chemical splash goggles. If hands become contaminated, wash immediately with undiluted bleach followed by soap and water.

Clean areas and materials contaminated by any aflatoxin solution or positive (i.e., > 20 ppb) extraction solutions spills with bleach. The affected area should be completely covered with 5-6 percent sodium hypochlorite (household bleach) dispensed from a plastic wash bottle or spray bottle. Apply 10 parts of bleach to 1 part of spilled material and leave for at least 5 minutes. Wipe up the bleach using an absorbent cloth or paper towels. Place cleaning materials in a plastic waste bag, close tightly, and discard.

2.7 DISPOSING EXCESS SAMPLE EXTRACT

a. Negative Samples (≤ 20 ppb).

Dispose of the excess sample extract solution in an approved waste container. Place the sample slurry in a garbage bag for routine disposal.

b. Positive Samples (>20 ppb).

The sample extract and slurry left over after completion of the test procedure must be decontaminated prior to disposal in a waste drum. Once the sample analysis has been completed, using a plastic wash bottle, add bleach to the slurry and allow to filter. When the filtration is complete, dispose of the slurry in a garbage bag.

To decontaminate the sample extraction solution, add bleach equal to one half of the volume remaining in the test tube. Pour the decontaminated extraction solution into an approved waste container.

2.8 WASTE DISPOSAL

Proper disposal of hazardous waste is required by law. The Environmental Protection Agency (EPA) establishes specific guidelines; however, additional local and State laws exist in some locations. It is important that the procedures used for disposing of waste chemicals comply with the laws required at each location.

Contact the local EPA office for disposal information and names of certified waste disposal companies in the area.

a. Chemicals and Solvents.

- (1) Dispose of waste according to existing local, State, and Federal laws.
- (2) Select an EPA approved or certified waste disposal company in the area. The company must be able to identify the type of waste drum required, provide information regarding sample profile and waste manifest requirements, and provide estimated costs for pick-up based on the results of the sample profile.
- (3) Locate waste drums in an area outside the laboratory space that complies with local fire and EPA codes. Label and date waste drums properly.
- (4) Post disposal procedures at each laboratory site.

- (5) Maintain accurate records with documentation from the disposal company of pick-up and delivery of the waste drums to the waste disposal site.

b. Decontaminated Materials.

Place decontaminated materials, such as filters, test kit components, and disposable lab materials into a garbage bag and dispose of in a dumpster or landfill disposal site. Only the materials that have been decontaminated may be transported. (Do not transport flammables or contaminated materials.)

c. Other.

Label excess ground corn/other grains remaining after aflatoxin testing and ground corn/other grains from official aflatoxin file samples representing grain with greater than 20 ppb: "FOR LABORATORY USE ONLY - NOT FOR USE AS FOOD OR FEEDSTUFF" and dispose of in a dumpster or landfill site.

2.9 FIELD OFFICE MANAGER RESPONSIBILITY

- a. Supplement this handbook with an SOP for each testing laboratory. The SOP should be tailored to accommodate the individual workload and environment for each location.
- b. Develop a Hazard Communication Program for personnel that perform tests involving hazardous materials and ensure that all personnel complete the program.
- c. Contact an EPA-approved or EPA-certified waste disposal company and make arrangements for removal of chemical wastes or provide other suitable waste disposal procedures consistent with existing laws that do not create a hazard to the community.
- d. Provide impermeable metal containers meeting Underwriters Laboratory approval for Class I liquids that can be tightly sealed and which are labeled "Flammable" or "Biohazardous Material" or both, as applicable, for storing waste chemicals (e.g., methanol, acetonitrile) and solutions for removal.
- e. Provide plastic disposal bags for disposal of decontaminated material such as filter paper, laboratory coats, disposable pipette tips, gloves, etc.

- f. Provide containers and labels for disposal of excess grain. Labels are to state "FOR LABORATORY USE ONLY - NOT FOR USE AS FOOD OR FEEDSTUFF," and are to be placed on containers prior to disposal.
- g. Provide signs for the laboratory door as follows:
 - (1) "Biohazardous Material Present."
 - (2) "No Smoking, Eating, or Drinking."
 - (3) "Flammable Material Present."
 - (4) "Wear Safety Protection."
 - (5) "Admittance of Authorized Personnel Only."
- h. Provide signs for the refrigerator, if present, as follows:
 - (1) "Biohazardous Material Present."
 - (2) "No Food or Drink to be Stored in This Refrigerator."
- i. Provide adequate training for laboratory employees prior to performance of laboratory functions to include:
 - (1) Information conveying operations and conditions which can result in exposure to aflatoxin.
 - (2) Contents and availability of Material Safety Data Sheets for relevant chemical agents.
 - (3) Precautions to take when working with aflatoxin contaminated products, including personal hygiene, personal protection equipment, and methods of decontamination.
 - (4) Purpose, proper care, and limitations of dust masks and other protective equipment.
 - (5) Engineering and work practice controls including cleaning methods.
 - (6) Review of the SOP at the laboratory.
 - (7) Proper handling and disposal of waste.

AFLATOXIN HANDBOOK

CHAPTER 2

3-4-02

j. Maintain the following safety and health records:

- (1) Records of any employee injury or illness involving over-exposure to chemicals (29 CFR 1904; 29 CFR 1960.66 through .77b).
- (2) List of employees trained and assigned to perform aflatoxin tests.
- (3) Copies of any safety and health studies pertaining to the laboratory.

1. D. 1990. 10. 10. 10. 10.

2. 1990. 10. 10. 10. 10.

3. 1990. 10. 10. 10. 10.

4. 1990. 10. 10. 10. 10.

5. 1990. 10. 10. 10. 10.

6. 1990. 10. 10. 10. 10.

7. 1990. 10. 10. 10. 10.

8. 1990. 10. 10. 10. 10.

9. 1990. 10. 10. 10. 10.

10. 1990. 10. 10. 10. 10.

11. 1990. 10. 10. 10. 10.

12. 1990. 10. 10. 10. 10.

13. 1990. 10. 10. 10. 10.

14. 1990. 10. 10. 10. 10.

15. 1990. 10. 10. 10. 10.

16. 1990. 10. 10. 10. 10.

17. 1990. 10. 10. 10. 10.

18. 1990. 10. 10. 10. 10.

19. 1990. 10. 10. 10. 10.

20. 1990. 10. 10. 10. 10.

21. 1990. 10. 10. 10. 10.

22. 1990. 10. 10. 10. 10.

23. 1990. 10. 10. 10. 10.

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, DC 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 3
3-4-02

CHAPTER 3

SAMPLE PREPARATION

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
3.1	GENERAL INFORMATION.....	3-1
3.2	SAMPLE SIZE	3-1
3.3	WORK RECORDS	3-1
3.4	SAMPLE PORTIONS	3-2
3.5	OPERATION OF GRINDERS	3-3
3.6	CLEANING GRINDERS	3-5
3.7	CHECKING PARTICLE SIZE	3-6

200 1/2 1/2
200 1/2 1/2
200 1/2 1/2

200 1/2 1/2

200 1/2 1/2

200 1/2 1/2

200 1/2 1/2

1/2

200 1/2 1/2

200 1/2 1/2

1/2

200 1/2 1/2

1/2

200 1/2 1/2

1/2

1/2

200 1/2 1/2

1/2

1/2

200 1/2 1/2

1/2

1/2

200 1/2 1/2

1/2

3.1 GENERAL INFORMATION

The manner in which samples are obtained and processed is an important consideration when testing for aflatoxin. To ensure that the test results accurately reflect the aflatoxin concentration present in a lot, samples must be representative of the lot and of sufficient size to compensate for the uneven distribution of the contaminant.

3.2 SAMPLE SIZE

Obtain samples according to the instruction in the Grain Inspection Handbook, Book I, "Grain Sampling."

The minimum sample size is based on the type of lot. Applicants may request a sample size larger than the minimum sample size.

Lot Type	Minimum Sample Size (lbs.)/ grams
Trucks	2 pounds / approximately 908 grams
Railcars	3 pounds / approximately 1,362 grams)
Barges/Sublots	10 pounds / approximately 4,540 grams

NOTE: A 10-pound sample size is also recommended, but not required, for submitted samples.

3.3 WORK RECORDS

Each testing laboratory must maintain work records for each test that include the name of the applicant, date of service, sample or carrier identification, test results, initials of official personnel performing the test, and any other information deemed necessary to properly certificate the test results and bill the applicant. As practical, use existing forms, such as FGIS-992, "Services Performed Report;" FGIS-920, "Grain Sample Ticket;" or FGIS-921, "Inspection Log," to record laboratory results.

Any sample sent to TSD (including the Board of Appeals and Review) for aflatoxin testing or monitoring purposes must include the necessary information to facilitate sample processing and testing.

3.4 SAMPLE PORTIONS

a. Subportions.

Grind the entire sample obtained for aflatoxin testing and prepare two 500-gram subportions from the ground sample: A 500-gram work portion for original testing services and a 500-gram file sample portion for review testing. For submitted samples, retain as large a sample as possible.

For subplot testing of corn at export locations, save an additional 500-gram file (three 500-gram subportions total) for Food and Drug Administration (FDA) analysis.

b. Saving File Samples.

Maintain file samples (including the FDA file sample when applicable) for all lots/samples that:

- do not meet the contractual specification of the applicant for service;
- are required for the aflatoxin monitoring program; or
- exceed FDA action limits of 20 ppb.

When applicable, maintain a representative file sample for each lot, subplot, composite, or submitted sample tested. For submitted samples that are less than 500 grams, retain as large a sample as possible. For information concerning file sample retention periods refer to FGIS Directive 9170.13, "Uniform File Sample Retention System".

c. Storing File Samples.

If file samples are required, store each sample in a manner that will maintain the representativeness of the sample and prevent possible manipulation or substitution. Place the sample in paper bags or envelopes and label each file sample with the test date and identification. Take precautions to ensure that file sample containers are strong enough to prevent loss of sample integrity when storing samples. Do not store samples near heat, windows, or in direct sunlight. (Store samples in cold storage if available.)

d. Disposition of File Samples.

At the end of the retention period, label the file samples as follows: "FOR LABORATORY USE ONLY - NOT FOR USE AS FOOD OR FEEDSTUFF," and discard the file samples in a dumpster or landfill disposal site.

e. Shipping Samples.

When it is necessary to send samples to other laboratory locations, take precautions to maintain sample integrity by securely packaging the samples. Label the shipping container "NOT FOR HUMAN CONSUMPTION".

3.5 OPERATION OF GRINDERS

Samples must be ground to a fine particle size that is sufficiently fine enough to obtain a homogeneous blend. Avoid over-grinding or pulverizing a sample because it produces an excessively powdery mix that will slow down the filtration process.

Grinding must be performed in an area separate from the testing area. Use the Romer Mill - Model 2A, Bunn Grinder, or equivalent to grind the sample.

FGIS employees must follow the manufacturer's safety procedures for operating the grinder and must wear protective equipment (i.e., labcoat, mask, gloves, and hairnet) when grinding samples.

a. Romer Mill.

(1) General Operating Instructions.

The Romer Mill simultaneously grinds and subsamples corn at the rate of approximately 1 pound per minute. An adjustable restrictor door located above the collection chute varies the amount of ground sample allowed into the collection chute. Official personnel must adjust the grinder to obtain the required testing and file portions from the sample.

Adjust the grinder by locating the first line (far left) etched on the restrictor door. Position the door approximately 1/3 of the way between the first and second line. For a 10-pound sample, approximately 500 grams will be collected through the collection chute. Once the grinder is adjusted to obtain the 500-gram sample, mark the location of the setting. To increase the sample size, move the restrictor door to the left.

Samples with moisture content of 20 percent or more may cause the grinder motor to overheat and the breaker switch to release. If this occurs, allow the motor to cool and then set the grind lever to the coarsest setting by turning it counterclockwise. Do not grind high moisture samples on the fine grind setting.

(2) Grinding the Sample.

Grind the entire 10-pound sample with the grind lever set at the finest range.

Collect the 500-gram sample in a clean container and stir the sample with a clean spatula for about 30 seconds. Using a spoon or spatula dip out a 50-gram portion for analysis and weigh on an FGIS-approved scale.

If a composite sample is required in addition to the subplot-by-subplot analysis, adjust portion sizes as needed to obtain an adequate size composite and still maintain individual file samples. Obtain the composite sample from the ground subplot samples.

b. Bunn Grinder.

(1) General Operating Instructions.

The Bunn-O-Matic grinds corn at a rate of approximately 2 pounds per minute and has a holding capacity of approximately 3 to 4 pounds when fully closed. Official personnel must grind the entire sample (see section 2.2) and cut it down (using an FGIS-approved divider) to obtain the required testing and file portions from the sample.

Samples with high moisture content of 20 percent or more may cause the grinder motor to overheat and the breaker switch to release. If this occurs, allow the motor to cool and then set the grind lever to the coarse setting.

(2) Grinding Samples.

Grind the entire 10-pound sample with the grind lever set at the fine selection. Add 3 to 4 pounds at a time into the hopper until all 10 pounds are ground. If the grinder is experiencing difficulty (e.g., over-heating, bogging down) at the fine setting, change the setting to coarse. After grinding the remainder of the sample at the coarse setting, switch the setting back to fine. Collect the entire 10-pound portion and regrind at the fine setting.

Using an FGIS-approved divider, cut out a representative portion of 500 grams, place in a clean container, and stir the sample with a clean spatula for about 30 seconds. Using a spoon or spatula dip out a 50-gram portion for analysis and weigh on an FGIS-approved scale.

3.6 CLEANING GRINDERS

A small amount of ground sample will remain in the grinder after the total sample has been ground. To prevent the contamination of subsequent samples, clean the grinder using one of the following cleaning procedures:

a. If a vacuum cleaner is available.

After a sample has been ground and collected, with the unit turned on, use a vacuum cleaner with an attachment that will fit over the mouth of the chute. Place the attachment at the bottom of each chute for about 30 seconds. After all the chutes have been cleaned, turn the power off and prepare for the next sample.

b. If a vacuum cleaner is not available.

Clear the grinder by discarding a small portion (first 10 to 15 grams) of the next sample to be tested.

- (1) Pour the sample into the grinder and turn it on long enough to collect the first 10 to 15 grams.
- (2) Turn the power off, and discard the 10-15 grams ground sample.
- (3) Turn the power back on and finish grinding the sample to collect the remaining subsample for analysis.

3.7 CHECKING PARTICLE SIZE

a. Procedures for Checking the Performance of the Grinder.

For locations that perform mycotoxin testing on coarse (e.g., corn) and small grains, perform the check using a 100-gram sample portion of corn using the following procedures.

- (1) Grind a sample portion of approximately 100 grams of corn having a moisture content of 14.0 percent or less.
- (2) Weigh the entire portion that was ground.
- (3) Sieve the portion across a standard No. 20 wire woven sieve.
- (4) Weigh the portion that passed through the sieve.
- (5) Determine the percent of fine material, by weight, as follows:

Fines = weight from step (4) divided by the weight from step (2) X 100.

b. Optimum Particle Size.

The optimum range for particles of coarse and small grain passing through the No. 20 sieve is between 60 and 75 percent. Whenever the ground particles appear to be too coarse, or the results of a grinder check indicate that less than 50 percent of the ground portion passes through the No. 20 sieve, the grinder should be adjusted or repaired to meet the optimum range requirements.

Grinding apparatuses must be checked periodically to determine whether they are producing a final product that meets the particle size requirements as listed above. Official personnel shall determine the frequency of the checks based on a number of items that include visual observation of the ground product, number of samples ground since last check, and time (number of days) since the last check was performed. Record all particle check results in a convenient location for future reference purposes.

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 4
3-4-02

CHAPTER 4

CERTIFICATION

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
4.1	BACKGROUND	4-1
4.2	GENERAL PROCEDURES	4-1
4.3	STANDARD CERTIFICATION STATEMENTS	4-4
4.4	OPTIONAL STATEMENTS	4-5
4.5	REVIEW INSPECTION STATEMENTS	4-6

ADVISORY BOARD

W. G. HORN
Chairman
D. C. COOPER
Vice Chairman

SECRETARY

Dr. J. R. D. COOPER, M.D., F.R.C.P.
Chairman, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Dr. J. R. D. COOPER, M.D., F.R.C.P.
Chairman, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Dr. J. R. D. COOPER, M.D., F.R.C.P.

Dr. J. R. D. COOPER, M.D., F.R.C.P.
Chairman, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Dr. J. R. D. COOPER, M.D., F.R.C.P.

Dr. J. R. D. COOPER, M.D., F.R.C.P.
Chairman, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Dr. J. R. D. COOPER, M.D., F.R.C.P.
Chairman, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

Dr. J. R. D. COOPER, M.D., F.R.C.P.
Chairman, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

4.1 BACKGROUND

Testing performed on standardized grains (e.g., corn, wheat) is performed as an official criteria factor under the authority of the United States Grain Standards Act (USGSA), as amended. Testing performed on processed grain products (e.g., corn meal) and other commodities is provided under the authority of the Agricultural Marketing Act (AMA) of 1946, as amended.

Aflatoxin results are recorded on the pan ticket, worksheet, or loading log and in the remarks section of the certificate.

Certify aflatoxin test results on grain in accordance with the USGSA/AMA (as applicable) regulations.

Upon the request of the applicant, separate certificates may be issued for grade and for aflatoxin when both are determined on the same lot.

Sections 800.125 and 800.135 of the regulations under the USGSA permit a review inspection on either official grade/factors or official criteria. When requested, a review inspection for official grade or official factors and official criteria may be handled separately, even though both sets of results are reported on the same certificate. When official grade or official factors and official criteria are reported on the same certificate, the review inspection certificate shall show a statement indicating that the review results are for official grade, official factors, or official criteria, and that all other results are those of the original, reinspection, or appeal inspection results, whichever is applicable.

4.2 GENERAL PROCEDURES

The type of service requested and the test method used determine how aflatoxin results are recorded and certified.

a. Qualitative Testing.

- (1) Record the results of a **qualitative service** on the pan ticket and inspection log as being equal to or less than a threshold (e.g., 20 ppb) or as exceeding the threshold.
- (2) If a **quantitative method** is used to provide qualitative service, record the quantitative test results on the work records even though the results are certified as meeting or exceeding a threshold.

(3) Certify results as being equal to or less than a threshold.

b. Quantitative Testing.

Record the results on the pan ticket and the inspection log to the nearest whole ppb.

When test results indicate that aflatoxin is present at a level of less than 5 ppb, certify the results as "Aflatoxin does not exceed 5 ppb."

Certify test results that are between 5 ppb and the conformance limit (e.g., 300 ppb) to the nearest whole ppb.

Test results greater than the conformance limit are certified as exceeding the conformance limit. For example: An aflatoxin test result of 410 ppb obtained using an aflatoxin test kit with a conformance limit of 300 ppb would result in the following certification statement: "Aflatoxin exceeds 300 ppb."

c. Certifying Test Results of Single and Combined Lots, Unit Trains, and Shiplots.

(1) Single lot inspection basis for trucks and railcars.

Certify each test result on a separate certificate.

(2) Combined land carrier basis for trucks and railcars.

If an applicant requests aflatoxin testing on a composite basis (up to 5 railcars and 15 trucks) and the inspection for grade on the basis of individual carriers, factor only certificates are issued for the aflatoxin testing and separate grade certificates are issued for each carrier.

(3) Composite Sample Testing for Shiplots.

Certify the composite results using the appropriate statement.

(4) Submitted Sample Testing.

Certify the results using the appropriate statement.

(5) Unit Train and Shiplot Inspection under the CuSum Loading Plan.

(a) Sublot Size for Shiplots.

The testing frequency for shiplot grain will be the same as the sample for grade analysis unless the applicant specifically requests aflatoxin analysis on the basis of a component sample.

(b) Sublot Size for Unit Trains.

The maximum size subplot for aflatoxin testing is 5 railcars for unit trains consisting of less than 200,000 bushels, or less than 50 cars. For unit trains consisting of 200,00 bushels or more, or 50 railcars or more, the maximum subplot size is 10 railcars.

For unit trains, the subplot size for aflatoxin testing and for grade analysis may be different. For example, an applicant may request grade analysis on the basis of a subplot containing two cars and request aflatoxin analysis on the basis of five cars.

(c) Recording Test Results.

Aflatoxin test results of subplot samples taken throughout loading are recorded on the loading log. A material portion occurs if the subplot result exceeds the limit as specified in the load order.

(d) Certifying Test Results.

Certify the lot based on the mathematical/weighted average (as applicable) of the accepted subplot results.

Certify material portions separately.

(e) Material Portions.

If a material portion occurs, the applicant has the option of requesting a review inspection. Review inspection results replace previous results when determining if a material portion exists.

If a material portion designation due to aflatoxin is not removed by the review inspection process, the applicant may leave the material portion on board and receive a separate certificate; return the grain to the elevator; or discharge the material portion along with additional grain in common stowage equivalent to one half the material portion quantity.

4.3 STANDARD CERTIFICATION STATEMENTS

Use one of the applicable statements for certifying aflatoxin.

a. Qualitative Testing.

When aflatoxin results are equal to or less than a specific threshold (e.g., 20 ppb) ppb:

"Aflatoxin equal to or less than 20 ppb."

"Aflatoxin exceeds 20 ppb."

b. Quantitative Testing.

(1) When aflatoxin results are less than 5 ppb use the following statement.

"Aflatoxin does not exceed 5 ppb."

(2) When aflatoxin test results are between 5 ppb and the conformance limit (e.g., 300 ppb) to the nearest whole number in ppb.

"Aflatoxin (result rounded to the nearest whole number) ppb."

(3) When aflatoxin test results are greater than the conformance limit (e.g., 300 ppb).

"Aflatoxin exceeds (enter conformance limit) ppb."

c. Board Appeals.

Board Appeals performed by the High Performance Liquid Chromatography (HPLC) method are certified to the nearest whole number in ppb.

"Aflatoxin (record actual results to the nearest whole number) ppb.
Results based on High Performance Liquid Chromatography Method."

4.4 OPTIONAL STATEMENTS

a. Estimated Results.

At the request of the applicant, certify test results that exceed the conformance limit as "estimated" unless a supplemental analysis is performed.

"Aflatoxin (result) Estimated."

NOTE: Do not show "estimated" if solution was diluted and supplemental analysis performed.

b. Aflatoxin Not Detected.

At the request of the applicant, use the following statement when aflatoxin is not detected (0 ppb).

"Aflatoxin not detected."

NOTE: If subplot results are combined and averaged and the lot average is equal to 0 ppb, but an individual subplot result exceeds 0.0 ppb, the statement may not be used.

c. Converting to Parts per Million (ppm).

At the request of the applicant, convert and certify the ppb result to parts per million (ppm) using an approved statement. To convert ppb to ppm, divide the ppb result by 1000.

"(Actual ppb result) ppb is equivalent to (converted ppm results) ppm."

d. Converting to Milligrams (mg) per Kilogram (kg), or Micrograms (μg) per Kilogram (kg).

At the request of the applicant, convert and certify results in milligrams per kilogram (mg/kg) or micrograms per kilogram (μg/kg). Use the following equivalents to determine mg/Kg or μg /kg:

$$\text{ppm} = \text{mg/kg}$$

$$\text{ppb} = \mu\text{g /kg}$$

e. Multiple Results on the Same Certificate.

When certifying multiple aflatoxin results on the same certificate and the results are based on different sample types, the certificate must reflect the difference. As a guideline, the multiple results are shown as follows:

"Sublot sample results: Aflatoxin equal to or less than 20 ppb."

"Composite sample result: Aflatoxin 14 ppm."

f. Negative Result Statement.

At the request of the applicant, one of the following statements may precede the applicable standard statements when test results are equal to or less than 20 ppb.

"The aflatoxin result is negative." OR "Negative aflatoxin."

g. Type of Test Statement.

At the request of the applicant, use this statement to indicate the type of aflatoxin test used.

"Results based on (indicate type of test used) method."

NOTE: These certification statements may be modified as deemed necessary.

4.5 REVIEW INSPECTION STATEMENTS

Use the appropriate statements listed below for reinspection, appeal, and Board appeal inspections.

- a. Results are reported on the same kind of certificate issued for the original service and supersede the previously issued inspection certificate.

"This certificate supersedes Certificate No. (number) dated (date)."

- b. The superseded certificate is null and void as of the date of the subsequent (reinspection/appeal/Board appeal) certificate.

"The superseded certificate has not been surrendered."

AFLATOXIN HANDBOOK
CHAPTER 4
3-4-02

- c. When a file sample is used, enter the following statement on the reinspection/appeal/Board appeal certificate:

"Results based on file sample."

- d. When reporting more than one official result on the same certificate but at different levels of inspection, explain this condition using one of the following applicable statements:

"(Grade, factor, or official criteria) results based on (new/file) sample. All other results are those of the original inspection service."

"(Grade, factor, or official criteria) results based on the appeal inspection. All other results are those of the (original inspection/reinspection) service."

"(Grade, factor, or official criteria) results based on the Board appeal inspection. All other results are those of the (original inspection/reinspection/appeal inspection) service."



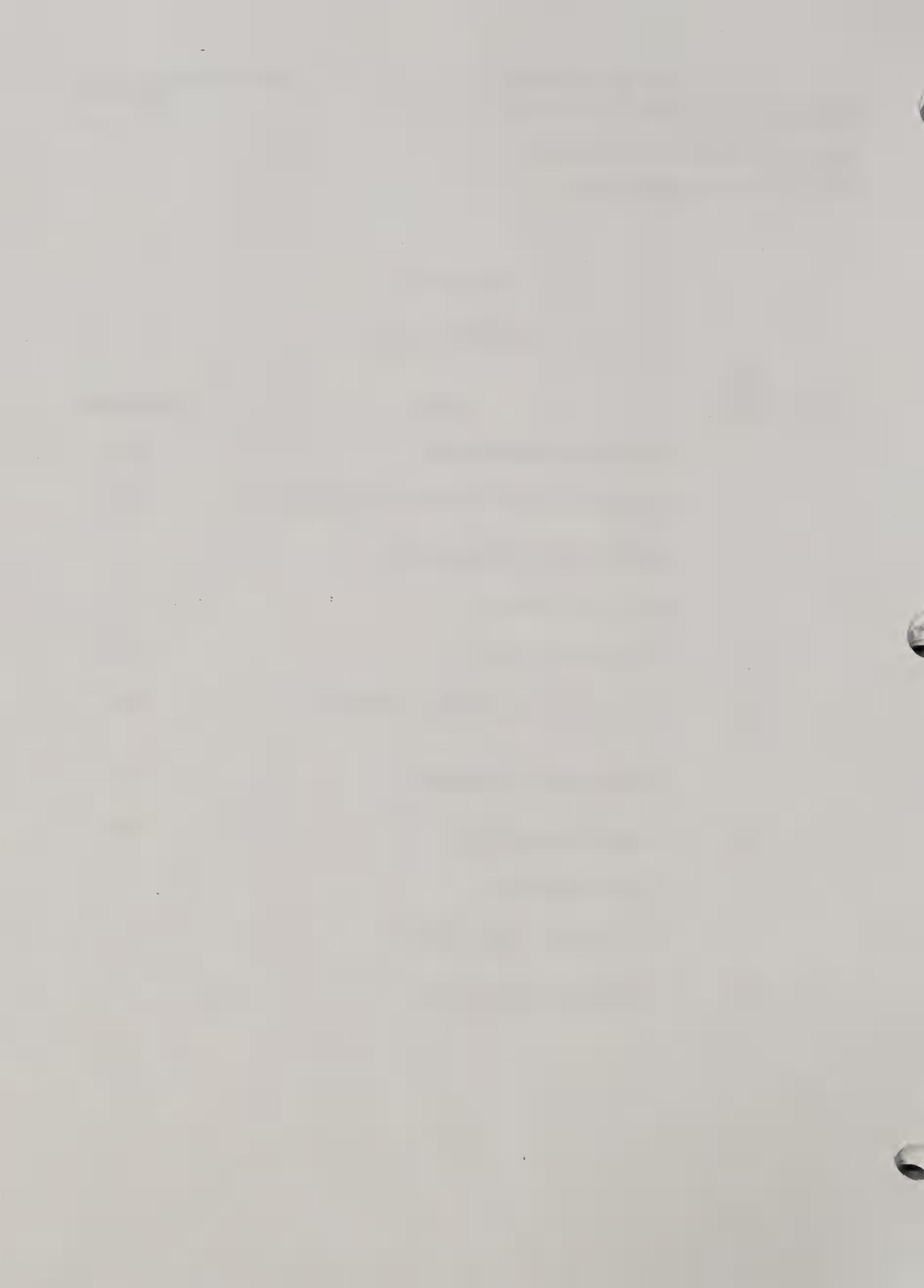
U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, DC 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 5
3-4-02

CHAPTER 5

AFLACUP TEST KIT

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
5.1	GENERAL INFORMATION	5-1
5.2	PREPARATION OF EXTRACTION SOLUTION	5-1
5.3	EXTRACTION PROCEDURES	5-1
5.4	REAGENT CHECK	5-2
5.5	TEST PROCEDURES	5-3
5.6	REPORTING AND CERTIFYING TEST RESULTS.....	5-5
5.7	HIGHER CUT-OFF POINTS	5-5
5.8	CLEANING LABWARE	5-6
5.9	WASTE DISPOSAL	5-7
5.10	EQUIPMENT AND SUPPLIES	5-7
5.11	STORAGE CONDITIONS	5-8



5.1 GENERAL INFORMATION

The AflaCup test kit is an enzyme linked immunosorbent assay (ELISA) developed by International Diagnostics Systems Corporation and marketed by Romer Labs. As the name implies, antibodies which react specifically with aflatoxins are contained in a cup. The test provides qualitative (less than or equal to a specified threshold) results.

5.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the AflaCup test method is a methanol/water (distilled or deionized) mixture consisting of 80 percent methanol (Reagent grade or better) and 20 percent water.

- a. Using a graduated cylinder, measure 800 ml of methanol and place it into a clean carboy with spigot.
- b. Add 200 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.
- c. Label the container stating the mixture (80 percent methanol and 20 percent water), date of preparation, and initials of technician who prepared the solution.
- d. Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 8 parts methanol to 2 parts of deionized or distilled water.

5.3 EXTRACTION PROCEDURES

- a. Transfer 50 grams of ground sample into an extraction mixing jar.
- b. Add 100 ml of the (80/20) methanol/water extraction solvent.
- c. Cover the extraction jar and blend on high speed for 1 minute.
- d. Remove the cover and funnel the extract through a Whatman No.1 filter or a coffee filter into a sample jar labeled with the sample identification.

- e. After collecting the filtrate, remove the funnel, filter, and ground material and place over an empty collection container.

5.4 REAGENT CHECK

- a. Stabilization.

Prior to performing the test, allow one hour for all reagents to reach room temperature (73° - 84°F).

- b. Testing Reagents.

Each day, before testing official samples, test at least one negative control cup to ensure that all reagents are functional.

Use the following procedures to test the control:

- (1) Apply 2 drops of negative control (green cap) to the center of the AflaCup.
- (2) Using a timer, allow the cup to set for a 1-minute reaction time.
- (3) Apply 2 drops of the aflatoxin enzyme (red cap) to the center of the cup.

Note: The enzyme solution may only be used with the antibody-coated cups contained in the same test kit.

- (4) Using a timer, allow the cup to set for a 1-minute reaction time.
- (5) Wash with 30 drops of the Wash Solution (white cap). When using more than one AflaCup, wash each cup with 3 series of 10 drops per cup.
- (6) Prepare fresh Substrate Solution in a small test tube by mixing 10 drops of Substrate Solution A (yellow cap) with 10 drops of Substrate Solution B (blue cap) for each AflaCup.

(Do not combine Substrate Solution A with Substrate Solution B more than 10 minutes before use.)

Note: If a blue color develops immediately after combining Substrates A and B, repeat this step. If the problem persists call Romer Labs for technical assistance.

- (7) Add the entire contents of the Substrate Mix from each test tube to each test cup in use.
- (8) Using a timer, allow the cup to set for a 1-minute reaction time.
- (9) Immediately read and interpret the result.

c. Interpreting Results.

A blue color indicates the reagents are functional. If the color remains white for at least one minute the reagents are not functional and must be replaced.

5.5 TEST PROCEDURES

a. Procedures for a 20 ppb cut-off.

- (1) Transfer 200 microliters (μl) of the dilution buffer to a culture tube (12x 75 mm) and then add 100 μl of the filtered extract.
- (2) Mix well and slowly apply 100 μl of the mixture to the center of the AflaCup.
- (3) Using a timer, allow the cup to set for a 1-minute reaction time.
- (4) Apply 2 drops of the aflatoxin enzyme (red cap) to the center of the cup.

Note: The enzyme solution may only be used with the antibody-coated cups contained in the same test kit.

- (5) Using a timer, allow the cup to set for a 1-minute reaction time.
- (6) Wash with 30 drops of the Wash Solution (white cap). When using more than one AflaCup, wash each cup with 3 series of 10 drops per cup.
- (7) Prepare fresh Substrate Solution in a small test tube by mixing 10 drops of Substrate Solution A (yellow cap) with 10 drops of Substrate Solution B (blue cap) for each AflaCup.

(Do not combine Substrate Solution A with Substrate Solution B more than 10 minutes before use.)

Note: If a blue color develops immediately after combining Substrates A and B, repeat this step. If the problem persists call Romer Labs for technical assistance.

- (8) Add the entire contents of the Substrate Mix from each test tube to each test cup in use.
- (9) Using a timer, allow the cup to set for a 1-minute reaction time.

b. Procedures for a 10 ppb cut-off.

- (1) Transfer 400 microliters (μ l) of the dilution buffer to a culture tube (12x 75 mm) and then add 200 μ l of the filtered extract.
- (2) Mix well and slowly apply two 100 μ l portions of the mixture to the center of the AflaCup.
- (3) Using a timer, allow the cup to set for a 1-minute reaction time.
- (4) Apply 2 drops of the aflatoxin enzyme (red-cap) to the center of the cup.

Note: The enzyme solution may only be used with the antibody-coated cups contained in the same test kit.

- (5) Using a timer, allow the cup to set for a 1-minute reaction time.
- (6) Wash with 30 drops of the Wash Solution (white cap). When using more than one AflaCup, wash each cup with 3 series of 10 drops per cup.
- (7) Prepare fresh Substrate Solution in a small test tube by mixing 10 drops of Substrate Solution A (yellow cap) with 10 drops of Substrate Solution B (blue cap) for each AflaCup.

(Do not combine Substrate Solution A with Substrate Solution B more than 10 minutes before use.)

Note: If a blue color develops immediately after combining Substrates A and B, repeat this step. If the problem persists call Romer Labs for technical assistance.

- (8) Add the entire contents of the Substrate Mix from each test tube to each test cup in use.
 - (9) Using a timer, allow the cup to set for a 1-minute reaction time.
- c. Interpretation of Test Results.
- (1) Negative (equal to or less than 20 ppb).

The sample is considered equal to or less than 20 ppb when the cup color changes to blue.

Note: Color will be concentrated in the center of the cup.
 - (2) Positive (Greater than 20 ppb).

The sample is considered greater than 20 ppb when the cup color remains white for at least one minute.

5.6 REPORTING AND CERTIFYING TEST RESULTS

- a. Report results on the pan ticket and inspection log as being equal to or less than a threshold (e.g., 20 ppb) or as exceeding the threshold.
- b. Certify results as being equal to or less than a threshold.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

5.7 HIGHER CUT-OFF POINTS

The manufacturer recommends the following procedures to obtain higher cut-off points. If the operator wishes to use higher than 20 ppb cut-off points, the following dilution procedures can be used with the dilution buffer provided. After making the dilution, the amount of sample applied to the AflaCup would remain at 100 µl.

Desired Sample Cut-off	Extract Volume into Dilution Tube	Dilution Buffer Used	Dilution Ratio
30 ppb	100 µl	350 µl	1:4.5
40 ppb	100 µl	500 µl	1:6.0
50 ppb	100 µl	650 µl	1:7.5
60 ppb	100 µl	800 µl	1:9.0
70 ppb	100 µl	950 µl	1:10.5
80 ppb	100 µl	1100 µl	1:12.0
90 ppb	100 µl	1250 µl	1:13.5
100 ppb	100 µl	1400 µl	1:15.0
200 ppb	100 µl	2900 µl	1:30.0
300 ppb	100 µl	4400 µl	1:45.0

5.8 CLEANING LABWARE

a. Negative Tests (\leq 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests ($>$ 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

5.9 WASTE DISPOSALa. Negative Results (\leq 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results ($>$ 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

5.10 EQUIPMENT AND SUPPLIESa. Materials Supplied in Test Kits:

- (1) Cups with Aflatoxin Antibody attached.
- (2) Aflatoxin Enzyme, Dropper Bottle.
- (3) Negative Control Solution, Dropper Bottle.
- (4) Wash Solution, Dropper Bottle.
- (5) Substrate A, Dropper Bottle.

- (6) Substrate B, Dropper Bottle.
- (7) Dilution Buffer for Samples, Translucent.

b. Materials Required but not Provided:

- (1) Sample grinder.
- (2) Balance.
- (3) Methanol - Reagent grade or better.
- (4) Distilled or deionized water.
- (5) Blender with mixing jars.
- (6) Cuvette rack.
- (7) Pipettor and tips - 100 to 1000 µl adjustable.
- (8) 100 ml graduated cylinder.
- (9) Funnel.
- (10) Timer.
- (11) Whatman No.1 Filter Paper or Coffee Filters.
- (12) Glass cuvettes (12 x 75 mm).

5.11 STORAGE CONDITIONS

Test kits should be refrigerated between 36°- 46°F.

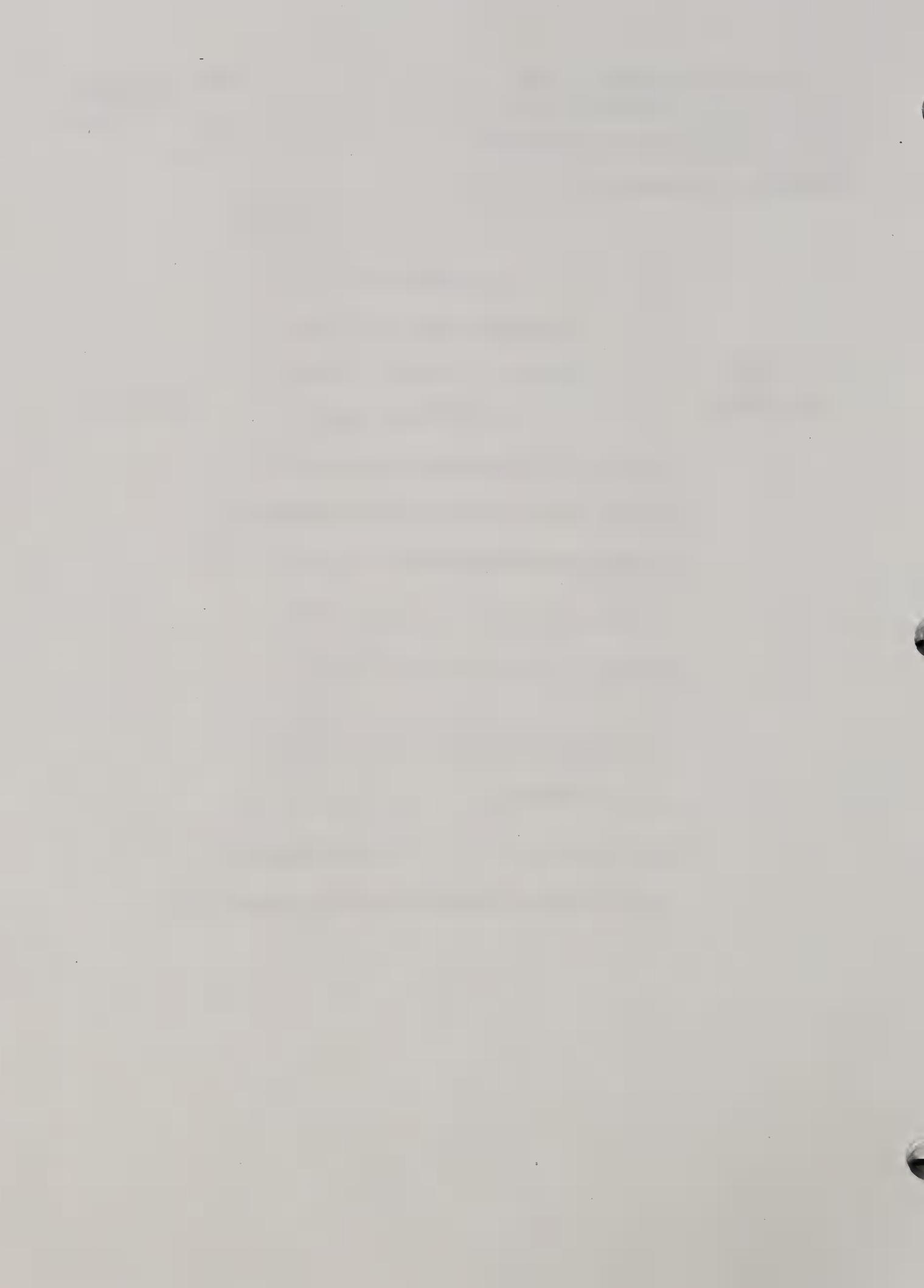
U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, DC 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 6
3-4-02

CHAPTER 6

EZ-SCREEN TEST KIT

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
6.1	GENERAL INFORMATION	6-1
6.2	PREPARATION OF EXTRACTION SOLUTION ...	6-1
6.3	EXTRACTION PROCEDURES	6-1
6.4	TEST PROCEDURES	6-2
6.5	REPORTING AND CERTIFYING TEST RESULTS.....	6-4
6.6	CLEANING LABWARE	6-5
6.7	WASTE DISPOSAL.....	6-5
6.8	EQUIPMENT AND SUPPLIES.....	6-6
6.9	STORAGE CONDITIONS.....	6-7



6.1 GENERAL INFORMATION

The EZ-Screen test is a sequential competitive enzyme immunoassay that provides qualitative (equal to or less than a specified threshold) results.

6.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the EZ-Screen test method is a methanol/water (distilled or deionized) mixture consisting of 80 percent methanol (Reagent grade or better) and 20 percent water.

- a. Using a graduated cylinder, measure 800 ml of methanol and place it into a clean carboy with spigot.
- b. Add 200 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.
- c. Label the container stating the mixture (80 percent methanol and 20 percent water), date of preparation, and initials of technician who prepared the solution.
- d. Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 8 parts methanol to 2 parts of deionized or distilled water.

6.3 EXTRACTION PROCEDURES

- a. Transfer 50 grams of ground sample into an extraction mixing jar.
- b. Add 100 ml of the (80/20) methanol/water extraction solvent.
- c. Cover the extraction jar and blend on high speed for 1 minute.
- d. Remove the cover and funnel the extract through a Whatman No.1 filter or a coffee filter into a sample jar labeled with the sample identification.
- e. After collecting the filtrate, remove the funnel, filter, and ground material and place over an empty collection container.

6.4 TEST PROCEDURES

a. Preparation of Solutions.

- (1) Select a test kit that has warmed to room temperature. Remove a 2 ml Dilution Buffer Tube from the kit and write the sample identification on the tube. Transfer 1 ml of the methanol extract from the sample to be tested to the tube and replace the cap. Shake the dilution buffer tube to mix the sample extract thoroughly.
- (2) Using a felt tipped pen, label a test card with the sample number and the date. Do not mark the card closer than 1/2 inch from the ports. Do not touch the test sites labeled "CONTROL and SAMPLE."
- (3) Prepare the Negative Control by removing the plastic shrink seal from around the dropper cap.
- (4) Prepare the Enzyme by squeezing the plastic dropper tube to crush the inner glass ampule. Use caution to ensure that the inner glass does not penetrate the outer plastic dropper tube. Tilt the tube back and forth for approximately 20 seconds to rehydrate and mix the contents. Do not shake vigorously or the contents will foam. Remove the plastic shrink seal from around the dropper cap.
- (5) Prepare the Substrate by squeezing the plastic dropper tube to crush the inner glass ampule. Shake the tube vigorously for approximately 20 seconds to rehydrate and mix the contents. Remove the plastic shrink seal from around the dropper cap.

b. Sample Analysis.

- (1) Place the test card on a clean, flat surface.
- (2) Using a 50 μ l pipette with a clean yellow tip attached, apply 50 μ l of the diluted sample extract to the card site labeled SAMPLE. Do not touch the pipette tip to the port. Hold the pipette so that the tip is approximately 1/2 inch above the port and allow the drop to fall freely.
- (3) Apply one drop of Negative Control to the "Control" port of the card. Do not touch the dropper tip to the port. Hold the tube so that the tip is approximately 1/2 inch above the port and allow the drop to fall freely.

- (4) Allow the "Sample" and "Control" drops to absorb into the test ports before proceeding to the next step.
- (5) Hold the Enzyme tube with the tip downward. Dislodge trapped air bubbles by tapping the side of the tube or shaking the contents toward the tip. Discard the first drop of Enzyme and then apply 1 drop to both of the ports on the test card. Do not touch the dropper tip to the ports. Hold the tube so that the tip is approximate 1/2 inch above the ports and allow the drops to fall freely.
- (6) Allow the Enzyme drops to absorb into the test ports before proceeding to the next step.
- (7) Apply 1 drop of Negative Control to both ports on the test card. Do not touch the dropper tip to the ports. Hold the tube so that the tip is approximately 1/2 inch above the ports and allow the drops to fall freely.
- (8) Allow the drops to absorb into the test ports before proceeding to the next step.
- (9) Carefully remove the excess liquid from around the edge of both ports on the test card using a cotton swab or clean, absorbent tissue.

NOTE: DO NOT TOUCH THE PORTS DIRECTLY.

- (10) Hold the Substrate tube with the tip downward. Dislodge trapped air bubbles by tapping the side of the tube or shaking the contents toward the tip. Discard the first drop of Substrate and then apply 2 drops to both of the ports on the test card. Do not touch the dropper tip to the ports. Hold the tube so that the tip is approximately 1/2 inch above the ports and allow the drops to fall freely. Immediately set a timer for 5 minutes.
- (11) When the timer goes off, read the test results as follows:
 - (a) If there is excess liquid at the edge of the ports, carefully remove it with a cotton swab or clean, absorbent tissue prior to reading.
 - (b) If color is visible in both the "Control" and "Sample" ports before the end of the 5 minutes, the test may be read before the timer goes off.

- (c) Using adequate lighting, examine the test card while leaving it flat on the work surface.
- (d) The "Control" port must function properly in order to have a valid test result. If the "Control" port does not develop a readily detectable color (remains colorless), the test result is NOT VALID. Do not continue, repeat the entire procedure or contact TSD for assistance.

If the "Control" port develops a readily detectable color of grey-blue or blue, the test result is VALID. Proceed with interpretation of test sample.

c. Interpreting Results.

Interpret the test results as follows:

(1) Equal to or less than 20 ppb.

The sample is considered less than or equal to 20 ppb when the "Sample" port develops color (light grey, grey-blue, or blue) over the surface of the port. Even though the "Sample" port may not yield a color equivalent to the color of the "Control" port, the sample is still considered less than or equal to 20 ppb.

(2) Greater than 20 ppb.

The sample is considered greater than 20 ppb when the "Sample" port fails to develop color (remains colorless). If the "Sample" port develops a narrow band of light grey color at the edge of the port with the rest of the port remaining colorless, such samples are considered greater than 20 ppb.

6.5 REPORTING AND CERTIFYING TEST RESULTS

- a. Report results on the pan ticket and inspection log as being equal to or less than a threshold (e.g., 20 ppb) or as exceeding the threshold.
- b. Certify results as being equal to or less than a threshold.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

6.6 CLEANING LABWARE

a. Negative Tests (\leq 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests ($>$ 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

6.7 WASTE DISPOSAL

a. Negative Results (\leq 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

6.8 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits:

- (1) QuikCards - 2.
- (2) Enzyme - 1 dropper tube.
- (3) Negative Control - 1 dropper tube.
- (4) Substrate - 1 dropper tube.
- (5) Dilution Buffer - 2 tubes of 2 ml each.
- (6) Sample Pipettes - 2 (**DO NOT USE**).

b. Materials Required but not Provided:

- (1) Timer (5 minute capacity).
- (2) Eppendorf pipette (or equivalent) 50 μ l capacity.
- (3) Eppendorf pipette tips, yellow (or equivalent).
- (4) Felt tipped pens.
- (5) Cotton swabs.
- (6) Balance.
- (7) Sample Grinder.

- (8) Methanol - Reagent grade or better.
- (9) Deionized or Distilled Water.
- (10) Blender with mixing jars.
- (11) Whatman No.1 Filter Paper or Coffee Filters.

6.9 STORAGE CONDITIONS

a. Storage Conditions.

- (1) Test kits should be refrigerated between 36° - 48°F.
- (2) Do not freeze any of the kit components or expose reagents to temperatures greater than 95°F.

b. Precautions.

- (1) The kit in its original packaging can be used until the end of the month indicated on the label when stored under refrigeration from 36° to 48° F.
- (2) Remove kits from the refrigerator and allow them to stand at room temperature for a minimum of 1 hour prior to performing the test. Failure to allow test kits to reach room temperature prior to use may result in false positive results.
- (3) Do not open test kits until ready for use.
- (4) Do not allow test kits to remain out of the refrigerator for more than 8 hours. Return unopened kits to the refrigerator for further use. Establish procedures to ensure that unopened test kits are not repeatedly left at room temperature and returned to the refrigerator. Ideally, identify kits left from the previous day and use these first.
- (5) Do not use reagents from one kit lot number with reagents from a different kit lot number.
- (6) Do not use reagents after the expiration date.
- (7) Do not perform testing with more than two test cards at a time.

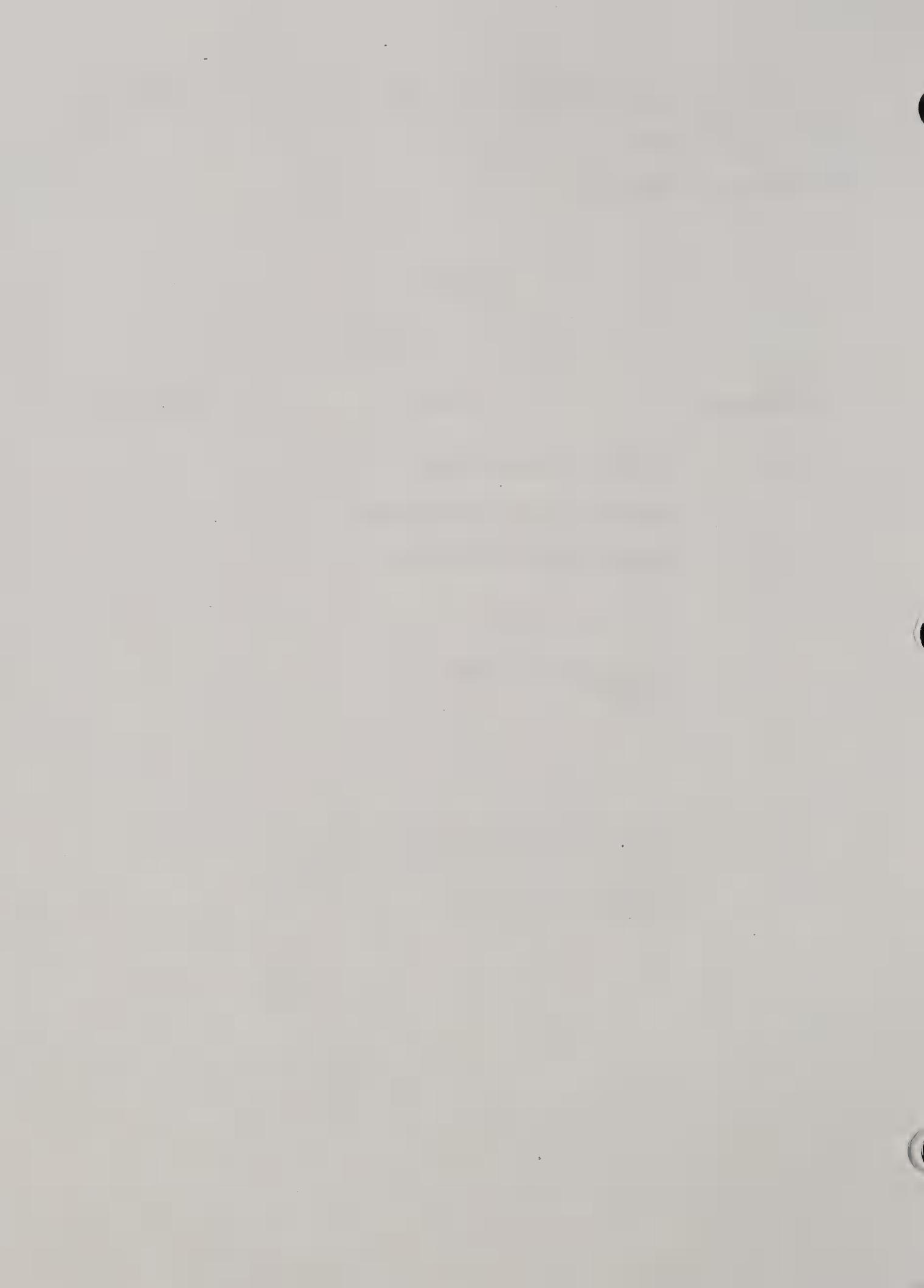
U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, DC 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 7
3-4-02

CHAPTER 7

AGRI-SCREEN TEST KIT

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
7.1	GENERAL INFORMATION	7-1
7.2	PREPARATION OF EXTRACTION SOLUTION ...	7-1
7.3	EXTRACTION PROCEDURES	7-1
7.4	TEST PROCEDURES	7-2
7.5	REPORTING AND CERTIFYING TEST RESULTS.....	7-5
7.6	CLEANING LABWARE	7-5
7.7	WASTE DISPOSAL.....	7-6
7.8	EQUIPMENT AND SUPPLIES.....	7-7
7.9	STORAGE CONDITIONS.....	7-8



7.1 GENERAL INFORMATION

The Agri-Screen test is a sequential competitive enzyme immunoassay that provides qualitative (equal to or less than a specified threshold) results.

7.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the Agri-Screen test method is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (ACS grade or better) and 30 percent water.

- a. Using a graduated cylinder, measure 700 ml of methanol and place it into a clean carboy with spigot.
- b. Add 300 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.
- c. Label the container stating the mixture (70 percent methanol and 30 percent water), date of preparation, and initials of technician who prepared the solution.
- d. Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 7 parts methanol to 3 parts of deionized or distilled water.

7.3 EXTRACTION PROCEDURES

a. Standard Procedure.

- (1) Transfer 50 grams of ground sample into an extraction mixing jar.
- (2) Add 250 ml of the (70/30) methanol/water extraction solvent.
- (3) Cover the extraction jar and blend on high speed for 2 minutes.
- (4) Remove the cover and funnel the extract through a Whatman No.1 filter or a coffee filter into a sample jar labeled with the sample identification.

- (5) After collecting the filtrate, remove the funnel, filter, and ground material and place over an empty collection container.
- b. Alternate Procedure.
- (1) Transfer 50 grams of ground sample into a whirlpack bag.
 - (2) Add 250 ml of the (70/30) methanol/water extraction solvent to the bag and secure tightly.
 - (3) Shake the sample portion and extraction solvent vigorously by hand for 3 minutes.
 - (4) Let the slurry stand for 1 minute, then pour off a small amount of the extract from the bag into the filter paper mounted over the collection container.
 - (5) Close the bag securely and save until ready for waste disposal.

7.4 TEST PROCEDURES

a. Preparation of Solutions.

- (1) Remove aluminum seals from blue-labeled, red-labeled, and yellow-labeled bottles and set aside.
- (2) Substrate is pre-activated and is ready for use. Substrate should be stored in the dark. Remove only one vial of substrate at a time from the foil pouch prior to use.

b. Sample Analysis.

- (1) Select a test kit that has stabilized/warmed to room temperature 68° - 82° F for 1-hour prior to use.
- (2) Open a foil bag and remove 2 red-marked mixing wells and 2 antibody-coated wells for each sample to be tested. Place in the microwell holder and mark the left end of the strip with a "1". Return unused strips to the foil package and close tightly.

Document the identification of all antibody-coated sample wells in order to identify the wells after washing.

	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
mixing wells	O	O	O	O	O	O	O	O	O	O	O	O
	C	S1	C	S2	C	S3	C	S4	C	S5	C	S6

"W" = well number e.g., #1 through #12

"C" = control

"S1, S2, S3, & S4" = sample numbers

Where C is the 20 ppb control, and S1, S2, etc. are the sample numbers

- (3) Firmly place a pipette tip on the 100 microliter (μ l) pipettor/syringe and add one preset amount of the Enzyme Conjugate (blue-labeled bottle) into each mixing well (priming pipette tip first). Discard pipette tip.

NOTE: When dispensing any test liquids, prime pipette tip by drawing liquid up into the tip and dispensing it back into the bottle one or two times.

- (4) Firmly place a pipette tip on the 100 μ l pipettor/syringe and add one preset amount from the 20 ppb Control (yellow-labeled bottle) to the first mixing well of the red-marked strip. Discard the pipette tip.

If testing more than one sample, place 100 μ l of 20 ppb Control into mixing well #3 (second sample), mixing well #5 (third sample), etc.

- (5) Firmly place a new pipette tip on the 100 μ l pipettor/syringe and add one preset amount from the filtered extract in the second well of the red-marked mixing strip. Discard tip.
- (6) Repeat step (5) for each subsequent sample using the designated sample wells and new pipette tip for each.

NOTE: If using a single channel pipettor or syringe, steps (5) through (8) must be performed individually and as quickly as possible.

- (7) Using a pipettor/syringe, mix the contents of the mixing wells by pipetting up and down in the tips 5 times.
- (8) Using a new tip for each well, transfer 100 μ l from each mixing well to the corresponding antibody-coated well. Discard red mixing wells and used pipette tips.
- (9) Mix the antibody-coated wells by gently sliding the microwell holder/wells back and forth on a horizontal surface for 10 to 20 seconds. Be careful not to splash solution out of wells.
- (10) Set the timer and allow the antibody-coated wells to incubate for 5 minutes.
- (11) After the incubation reaction is complete, shake out the contents of the antibody-coated wells.
- (12) Using a wash bottle, fill each antibody-coated well with distilled/deionized water and dump out. Repeat this step 10 times.
- (13) Remove all water droplets by turning wells upside down and gently tapping over a paper towel until all of the water is removed.
- (14) Firmly place a new pipette tip on the pipettor/syringe and transfer 100 μ l of substrate (green-capped tube) to each antibody-coated well. Discard tip.
- (15) Mix the antibody-coated wells by gently sliding the microwell holder/wells back and forth on a horizontal surface for 10 to 20 seconds. Be careful not to splash solution out of wells.
- (16) Set the timer and allow the antibody-coated wells to incubate for 5 minutes.
- (17) After the incubation reaction is complete, firmly place a new pipette tip on the pipettor/syringe and transfer 100 μ l of red stop solution (red-labeled bottle) to each antibody-coated well. Discard tip.
- (18) Mix the antibody-coated wells by gently sliding the microwell holder/wells back and forth on a horizontal surface for 10 to 20 seconds. Be careful not to splash solution out of wells.

c. Interpreting Results.

Place the well strip on a white surface when determining results. Interpret the test results as follows:

(1) Equal to or less than 20 ppb.

The sample is considered equal to or less than 20 ppb when the "Sample" well is as blue or darker (blue) than the control well.

(2) Greater than 20 ppb.

The sample is considered greater than 20 ppb when the "Sample" well shows less blue color (more red color) than the control well.

7.5 REPORTING AND CERTIFYING TEST RESULTS

- a. Report results on the pan ticket and inspection log as being equal to or less than a threshold (e.g., 20 ppb) or as exceeding the threshold.
- b. Certify results as being equal to or less than a threshold.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

7.6 CLEANING LABWARE

a. Negative Tests (\leq 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

7.7 WASTE DISPOSAL

a. Negative Results (\leq 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

7.8 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits

- (1) Foil pouch with 24 antibody-coated wells and 24 red mixing wells.
- (2) 1 yellow-labeled bottle of 20 ppb aflatoxin control solution.
- (3) 1 blue-labeled bottle of enzyme conjugate solution.
- (4) 1 green-labeled bottle of substrate solution.
- (5) 1 spring syringe.
- (6) 75 pipette tips.

b. Materials Required but not Provided:

- (1) Timer (5 minute capacity).
- (2) 100 µl pipettor (single or multi-channel) with tips.
- (3) Microwell holder.
- (4) Wash bottle.
- (5) Felt tipped pens.
- (6) Balance.
- (7) Sample Grinder.
- (8) Methanol - ACS grade or better.
- (9) Deionized or Distilled Water.
- (10) Blender with mixing jars.
- (11) Whatman No.1 Filter Paper or Coffee Filters.

7.9 STORAGE CONDITIONS

a. Storage Conditions.

- (1) Test kits should be refrigerated between 36°- 48°F.
- (2) Do not freeze any of the kit components or expose reagents to temperatures greater than 95° F.

b. Precautions.

- (1) Do not use kit components beyond expiration date.
- (2) Do not use reagents from one kit with reagents from a different kit.
- (3) Use of incubation times other than those specified may give inaccurate results.
- (4) Avoid prolonged storage of kits at ambient temperatures.

U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 8
3-4-02

CHAPTER 8

AFLATEST TEST METHOD

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
8.1	GENERAL INFORMATION.....	8-1
8.2	PREPARATION OF SOLUTIONS	8-1
8.3	FLUOROMETER CALIBRATION	8-2
8.4	SOLUTION TESTING	8-6
8.5	TEST PROCEDURES	8-7
8.6	CLEANING LABWARE	8-15
8.7	WASTE DISPOSAL	8-16
8.8	EQUIPMENT AND SUPPLIES	8-17
8.9	STORAGE CONDITIONS	8-18



8.1 GENERAL INFORMATION

The Aflatest method of testing for aflatoxin uses monoclonal antibody affinity chromatography that provides for quantitative measurement of total aflatoxins (B1, B2, G1, and G2) in parts per billion (ppb) or qualitative (screening) for aflatoxin.

8.2 PREPARATION OF SOLUTIONS

Prior to beginning test procedures, prepare the solutions required for testing. The distilled/deionized water, dilute developer solution, and the HPLC grade methanol must be checked for background fluorescence with the fluorometer after properly calibrated. None of the above reagents should give a positive reading of more than 1.0 ppb.

a. Dilute Developer Solution.

NOTE: Developer Solution must be prepared fresh daily.

The concentrated developer solution should have a slight reddish brown color. (Do not use the stock solution if it is colorless.) Loss of color indicates that the stock solution has lost its potency.

Prepare dilute developer solution by adding 5 ml of Aflatest developer concentrate (Vicam Cat. # 32010) to 45 ml of distilled/ deionized water. Mix well, and label the dilute developer solution bottle showing the date and time of preparation.

DO NOT USE IT AFTER 6 HOURS HAVE ELAPSED.

If the amount of dilute developer being prepared needs to be adjusted based on the workload at individual locations, make sure that the 1 part concentrated developer to 9 parts distilled/deionized water ratio is maintained.

Label each stock bottle of concentrated developer with the date on which it was first opened. **DO NOT USE IT AFTER 30 DAYS HAVE ELAPSED.**

b. 80/20 Percent Methanol Solution.

Make up the solution by using the ratio of 8 parts HPLC grade methanol to 2 parts deionized/distilled water. Prepare the 80 percent methanol water solution by adding 800 ml methanol to 200 ml of water. Mix well. Keep the bottle tightly capped when not in use.

Label the 80 percent methanol/water solution bottle showing date of preparation. If the amount of the 80 percent methanol solution being prepared needs to be adjusted based on the workload at individual locations, make sure that the 8 parts HPLC grade methanol to 2 parts distilled/deionized water ratio is maintained.

To prepare smaller or larger amounts of solution the ratio of 8 parts methanol to 2 parts of deionized or distilled water must be maintained. For example: To prepare a solution that will provide for 5 test extractions (100 ml per test sample) mix 400 ml HPLC grade methanol to 100 ml deionized or distilled water.

8.3 FLUOROMETER CALIBRATION

a. General.

An FGIS-approved fluorometer is used to determine the aflatoxin level. To ensure accurate results, calibrate the fluorometer prior to use each day and verify at least once an hour using the **Yellow Vial**.

Turn the fluorometer on with the On/Off switch located on the rear panel. When the fluorometer is turned on, allow it to warm up for 10 minutes before calibrating. Once the fluorometer is turned on, it may be left on until close of business for the day. If the fluorometer is turned off during the day, a 10-minute warm up is required.

After turning the fluorometer on, it will identify itself and perform a set of self-tests. The fluorometer will then proceed through a printer check. All self-tests and the printer check must pass or an error message is displayed. If any error message appears, consult the operator's manual.

(1) Date and Time.

When date and time are first shown, the display reads "DATE XX/XX/XX TIME XX:XX:XX." Use keypad to enter the correct date and time as follows:

- (a)** If date and time are correct, depress the arrow key under "CONTINUE" and proceed to step (2).

- (b) If the date and/or time need to be changed select the mode (date or time) and make the changes using the keypad. When the correct date and/or time are entered, depress the arrow key under "ENTER." The system displays the corrected information. Press the arrow key under "CONTINUE."

(2) Test Delay Time.

The default test delay time is 60 seconds, which is correct for the Aflatest-P assay. Press "ENTER."

(3) Measurement Units.

Set measurement units to ppb by pressing the arrow key under PPB. The fluorometer briefly displays "STARTING CALIBRATION, CHECKING GAINS, WAIT." Then the display message will change to "INSERT CALIBRATION VIAL."

b. Calibration Standards.

(1) Maintenance.

The standard solutions in the three (3) standard vials (Red, Green, and Yellow) degrade slowly in the presence of light.

Since the plastic case containing the vials passes a small amount of light, it is recommended that both case and vials be stored in a cabinet or drawer away from all light except when calibrating or checking the calibration of the fluorometer.

Maintain two (2) sets of standards (two cases) at each location. Select and identify one set as the working standard, the other as the reference standard to be used to check the working standard every 14 days.

The degradation of the working set will occur gradually over a period of time, so anticipate expiration and requisition a replacement set in advance. (A sudden change in the reading of a vial indicates instrument instability, a cracked vial, or undue exposure of the vial to light.)

When one vial of a set expires, replace the entire set. About 2 months before the expected expiration of the working set, obtain a new set of standards from Vicam Co. When received, compare fluorometer readings of the new set with those of the existing reference set. If the difference between the two sets exceeds 3 ppb for any of the colors, notify TSD.

(2) Biweekly check of working standards.

Calibrate the fluorometer using the working set as described in "Calibration Procedures" (see section 8.3 c).

After calibrating the working set, remove the reference set from storage and test the 3 vials as described in section 8.4, c, (8). The difference in readings of the two sets should not exceed the following limits:

<u>Red</u>	<u>Yellow</u>	<u>Green</u>
± 10 ppb	± 5 ppb	± 2 ppb

If the difference between the working and reference sets exceeds the tolerances, discard the working set. Begin using the old reference set as the working set, and use the new set as the reference set. Keep a permanent record of all calibration verification data.

c. Calibration Procedures

- (1) Turn on the fluorometer and warm it up for 10 minutes. Set date, time, test delay time, and measurement units. When the display shows "INSERT CALIBRATION VIAL," proceed to the next step.
- (2) Wipe the Red Vial with a clean cloth or paper wipe and insert it into the sample well. Be sure the vial is fully inserted and touches the bottom of the sample well.

The display will read "CALIBRATOR VALUE 20.0 PPB PRESS ENTER." The correct value for the red vial is 150 ppb rather than the default value of 20 ppb. Enter the correct value of 150 ppb.

- (3) Use the keypad to enter 150, and press "ENTER" to start the calibration sample measurement.

The display will read "MEASURING CALIBRATOR." When the calibration measurement is completed, the display will read "REMOVE CALIBRATION VIAL."

- (4) After removing the Red Vial, the display will read "INSERT BLANK VIAL, CONTINUE CALIBRATION."

Wipe the Green Vial with a clean cloth or paper wipe and then insert it into the sample well. Be sure the vial is fully inserted and touches the bottom of the sample well.

- (5) The display will read "BLANK VALUE 0.0 PPB, PRESS ENTER." The correct value for the green vial is -3 ppb rather than the default value of 0 ppb.

Enter the correct value of -3 ppb. (The value of -3 is an offset to correct for a small amount of material which bleeds from the column during the assay.) Use the keypad to enter -3, and press "ENTER" to start the blank measurement.

The display will read "MEASURING BLANK." When the blank measurement is completed, the message will read "MEASURING BLANK, REMOVE BLANK VIAL."

- (6) After removing the Green Vial, the display will read "PUSH ENTER TO CONTINUE, RECAL <SOFT KEYS> RETEST."

- (7) The calibration process is now completed.

Press "ENTER" to prepare the instrument for measuring unknown samples. The display will read "READY TO START TESTING, INSERT SAMPLE TO MEASURE."

- (8) Check the calibration by testing the Yellow Vial.

Wipe the vial with a clean soft lint free cloth or paper wipe and insert it into the sample well. Be sure the vial is fully inserted and touches the bottom of the sample well. The value displayed should be 75 ± 5 ppb. If it is not, repeat the calibration process (steps 2 through 7, above), then check the Yellow Vial again.

To restart the recalibration process, press the arrow on the left-hand side of the display. Record the result for the Yellow Vial. If the Yellow Vial still reads out of tolerance, contact the Mycotoxin Testing Group at TSD.

- (9) When the fluorometer is calibrated, place the standards back in the case and close tightly, and store away from any light source.
- (10) Check the calibration of the fluorometer at least once an hour or before analyzing any test samples if more than 1 hour time has elapsed since the last test using the Yellow Vial (75 +/- 5).

8.4 SOLUTION TESTING

The distilled/deionized water, dilute developer solution, and HPLC grade methanol must be tested for background fluorescence before use. After calibrating the fluorometer perform the following:

a. Methanol.

Place 2.0 ml of HPLC grade methanol into a clean cuvette. Place the cuvette in the calibrated fluorometer. The displayed reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, replace the methanol.

b. Water.

Dispense 2.0 ml of deionized/distilled water into a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, take action to assure a pure water supply.

c. Developer Solution.

Combine 1.0 ml of dilute developer solution and 1.0 ml of HPLC grade methanol in a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0.

If the reading is positive and greater than 1.0, check each reagent separately to determine which reagent is causing the problem and replace it.

8.5 TEST PROCEDURES

- a. Procedures for Testing Corn, Corn Meal, Corn/Soy Blend, Milled Rice, Popcorn, Sorghum, and Soybeans.

Note: All aflatoxin tests for rice are performed on a milled rice basis. Consequently, rough rice or brown rice require milling before analysis. Mill rough rice or brown rice according to the procedures in the Rice Handbook.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 100 ml of the 80/20 methanol/water extraction solution.
- (d) Cover jar and blend at high speed for 1 minute.
- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
- (f) Collect the filtrate in a clean beaker labeled with the sample identification.
- (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
- (h) Pipette 5 ml of filtered extract into a clean beaker.
- (i) Add 10 ml of distilled/deionized water and mix thoroughly.
- (j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.

- (k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
- (b) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.
- (c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

- (d) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.
- (e) Repeat the water wash in step (d) above.
- (f) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicam Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.

- (g) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.
 - (h) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.
 - (i) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).
 - (j) **Immediately** place the cuvette in a calibrated fluorometer.
- (3) Reading, Recording, and Certifying Test Results.
- (a) Record the digital readout (after 60 seconds) as ppb total aflatoxins in the sample.
 - (b) Report all results on the pan ticket and the inspection log to the nearest whole ppm.
 - (c) Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
 - (d) Refer to the Certification section of the handbook for more detailed certification procedures.
- (4) Supplemental Analysis.

To determine and report an aflatoxin level higher than 300 ppb, the filtered test sample extract must be diluted so that a value between 5 ppm and 300 ppm is obtained. The final aflatoxin concentration is calculated by multiplying the results obtained with the diluted extract by the dilution factor.

- (a) Using an Eppendorf pipette, add 0.5 ml (instead of 1.0 ml) of the filtered diluted extract to the top of the Aflatest column headspace. (See section 8.5 a (2) (b).)
- (b) Analyze the filtered extract as a normal sample.
- (c) Multiply the analytical results obtained by 2 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the sample value obtained using the diluted test sample procedure, the actual concentration in the original sample was 480 ppb.

Example:	Diluted test sample extract result	240 ppb
	Dilution factor	<u>x 2</u>
	Actual aflatoxin concentration	480 ppb

Note: Laboratories may dilute samples as a first step if levels typically observed exceed 300 ppb and the applicant requests actual (not estimated) readings.

b. Procedures for Testing Corn Germ Meal and Wheat.

- (1) Extraction.
 - (a) Place 50 g of ground sample into blender jar.
 - (b) Add 10 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
 - (c) Add 200 ml of the 80/20 methanol/water extraction solution.
 - (d) Cover jar and blend at high speed for 1 minute.
 - (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
 - (f) Collect the filtrate in a clean beaker labeled with the sample identification.
 - (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.

- (h) Pipette 5 ml of filtered extract into a clean beaker.

Note: If the solution filtration is slow (i.e., more than two minutes are required to collect 5 ml of filtrate), withdraw 5.0 ml of the clearest liquid from the top of the material held in the funnel (see step (e) above) and transfer it to a clean container.

- (i) Add 10 ml of distilled/deionized water and mix thoroughly.
- (j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
- (k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
- (b) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.
- (c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

- (d) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.

- (e) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.
 - (f) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.
 - (g) Repeat the water wash in step (f) listed above.
 - (h) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicam Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.
 - (i) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.
 - (j) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.
 - (k) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).
 - (l) Immediately place the cuvette in a calibrated fluorometer.
- (3) Reading, Recording, and Certifying Test Results.
- (a) Record the digital readout (after 60 seconds) as ppb total aflatoxins in the sample.

- (b) Report all results on the pan ticket and the inspection log to the nearest whole ppm.
- (c) Sample results over 300 ppb are reported as >300 unless a supplemental analysis is performed.
- (d) Refer to the Certification section of the handbook for more detailed certification procedures.

c. Procedures for Testing Corn Gluten Meal and Corn Gluten Feed.

(1) Fluorometer Calibration.

Calibrate fluorometer as follows:

Red Vial	110 ppb
Green Vial	-3 ppb
Yellow Vial	55 +/- 5ppb

(2) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 250 ml of 60 percent methanol/40 percent water extraction solution to blender jar. The 60/40 percent methanol/water solution is prepared by mixing 600 ml HPLC grade methanol with 400 ml distilled/deionized water.
- (d) Cover jar and blend at high speed for 1 minute.
- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
- (f) Collect the filtrate in a clean beaker labeled with the sample identification.

- (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
 - (h) Pipette 10 ml of filtered extract into a clean beaker.
 - (i) Add 20 ml of distilled/deionized water and mix thoroughly.
 - (j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
 - (k) Load 6-8 ml of the filtrate from step (j) above into a 10 ml plastic syringe barrel fitted with 0.22 micron nylon syringe disk filter (Fisher Scientific Corporation CAMEO II Cat. No. DDN 02T2550, Gelman Cat. No. 09-730-191, or Corning Cat. No. 09-754-22).
 - (l) Apply enough air pressure to syringe barrel to produce a flow of approximately 1 drop per second through disk filter and collect a minimum of 5 ml of filtrate in a clean test tube. Discard filter disk.
- (3) Affinity Column.
- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
 - (b) Using a 1.0 ml Eppendorf pipette, load 4.0 ml of refiltered extract from step (l) above into the barrel of a 10 ml glass syringe to which an Aflatest-P column is attached.
 - (c) Apply pressure so that the extract passes through the column at 1 to 2 drops per second. Remove syringe barrel from column. Fill column with distilled water. Reattach syringe barrel to column.
 - (d) Fill syringe barrel with 10 ml of distilled/deionized water and pass through column at a flow rate of approximately 2 drops per second. Allow all of wash water to pass through column.
 - (e) Repeat column wash with another 10 ml of deionized/distilled water.

- (f) Elute aflatoxin from Aflatest-P column with 1 ml HPLC grade methanol and collect sample eluate solution in glass cuvette.
 - (g) Add 1 ml of fresh, dilute Aflatest developer solution directly to the eluate in cuvette and mix well.
 - (h) **Immediately** place the cuvette in a calibrated fluorometer.
- (4) **Reading, Recording and Certifying Test Results.**
- (a) Record the digital readout (after 60 seconds) as ppb total aflatoxins in the sample.
 - (b) Report all results on the pan ticket and the inspection log to the nearest whole ppm.
 - (c) Sample results over 300 ppb are reported as >300 ppb.
 - (d) Refer to the Certification section of the handbook for more detailed certification procedures.

NOTE: Rinse both glass and plastic syringe barrels with approximately 10 ml of distilled/deionized water each before analyzing next sample.

8.6 CLEANING LABWARE

- a. **Negative Tests (\leq 20 ppb).**

- (1) **Labware.**

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

- (2) **Disposable Materials.**

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

8.7 WASTE DISPOSAL

a. Negative Results (\leq 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

8.8 EQUIPMENT and SUPPLIES

- a. Fluorometer - Romer model RL-100, Vicam Series III and IV, or Vicam model MF-2000.
- b. Fluorometer calibration standards. (Vicam # 33050)
- c. Cuvette Rack. (Vicam # 21010)
- d. Pump assembly stand, double. (Vicam # 21030)
- e. Syringe, glass 10 ml. (Vicam # 34010)
- f. Syringe hand pump with coupling. (Vicam #36030)
- g. Automatic pipettor (1 ml capacity for methanol). (Vicam #20501)
- h. Automatic pipettor (1 ml capacity for developer). (Vicam #20600)
- i. Graduated cylinders - 25 ml, 100 ml, and 250 ml capacity.
- j. Aflatest-P columns. (Vicam # 12022)
- k. Cuvettes, disposable 12 x 75 mm borosilicate glass tube. (Vicam # 34000)
- l. Disposable beakers. (Vicam # 36010)
- m. Glass microfibre filter paper -Whatman 934-AH. (Vicam # 31955)
- n. Small plastic funnels.
- o. Wash bottles or spray bottles.
- p. Box of Kim Wipes (small size sheets).
- q. HPLC grade methanol.
- r. Aflatest developer solution. (Vicam # 32010)

- s. Balance.
- t. Sample Grinder.
- u. Distilled/deionized water.
- v. Aflatest developer solution. (Vicam #32010)
- w. USP grade sodium chloride (NaCl) or food grade un-iodized salt.

8.9 STORAGE CONDITIONS

- a. Affinity Columns - Store at room temperature (64° to 86° F).
- b. Calibration Vials - Store in a cabinet or drawer away from all light, except when in use.
- c. Developer Concentrate - Store in a tightly closed bottle in a cool, dry, well ventilated area and away from sunlight, combustible materials, and incompatible materials.

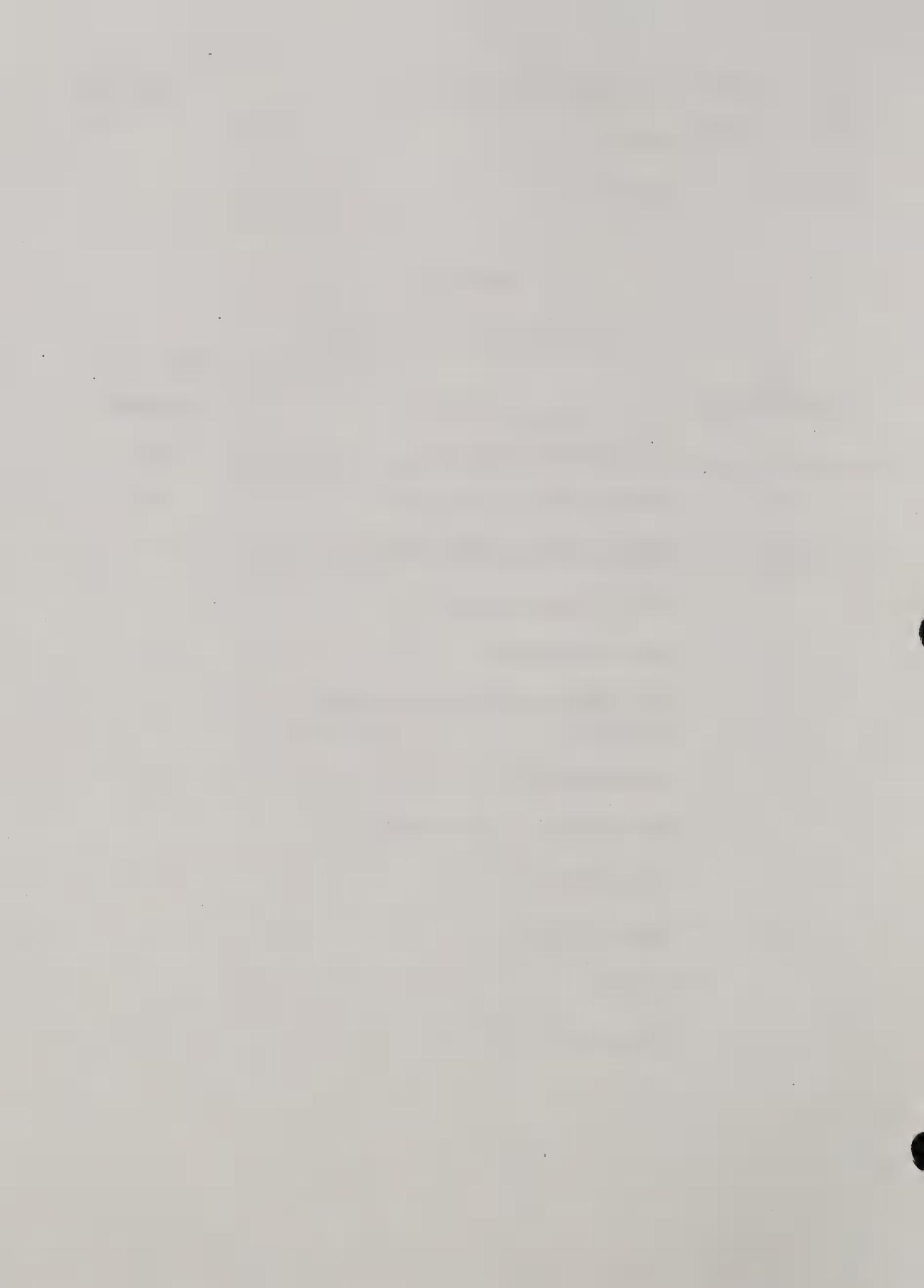
U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, D.C. 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 9
3-4-02

CHAPTER 9

FLUOROQUANT TEST METHOD

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
9.1	GENERAL INFORMATION.....	9-1
9.2	PREPARATION OF SOLUTIONS	9-1
9.3	FLUOROMETER CALIBRATION	9-2
9.4	EXTRACTION PROCEDURES	9-3
9.5	TEST PROCEDURES	9-4
9.6	REPORTING AND CERTIFYING TEST RESULTS	9-6
9.7	SYSTEM CHECK	9-6
9.8	SUPPLEMENTAL ANALYSIS	9-7
9.9	CLEANING LABWARE	9-8
9.10	WASTE DISPOSAL	9-9
9.11	EQUIPMENT AND SUPPLIES	9-9
9.12	STORAGE CONDITIONS	9-10



9.1 GENERAL INFORMATION

The Romer Fluoroquant aflatoxin test method uses fluorescence technology to quantitatively measure total aflatoxins (B1, B2, G1, and G2) in parts per billion (ppb).

9.2 PREPARATION OF SOLUTIONS

a. Developer Solution.

Prepare the working developer reagent by adding 50 ml of deionized or distilled water to the bottle supplied with the repipettor and then adding the contents of one ampule of developer concentrate.

To insure complete and accurate transfer, rinse the ampule three times with the working developer solution, each time returning the rinse to the bottle. Swirl to mix contents.

There is room in the repipettor bottle to make a larger amount of developer by adding the contents of 2 ampules to 100 ml of water.

Initially prime the repipettor in a waste tube to remove any air bubbles. When using the repipettor, pull up the plunger all the way to the stop, then push all the way back down to insure accurate delivery volume. Before each use, prime the repipettor slightly to remove any air that may form at the tip. Place the cap on the end of the repipettor tip after each use.

NOTE: The developer working reagent must be made fresh every 8 hours.

- b. 80 Percent Methanol Solution (for corn, corn meal, rice, popcorn, sorghum, and wheat).
- (1) Using a graduated cylinder, measure 800 ml of methanol (HPLC grade) and place it into a clean carboy with spigot.
 - (2) Add 200 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.
 - (3) Label the container stating the mixture (80 percent methanol and 20 percent water), date of preparation, and initials of technician who prepared the solution.

- (4) Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 8 parts methanol to 2 parts of deionized or distilled water.

- c. **90 Percent Acetonitrile Solution (for soybeans and corn/soy blend).**
 - (1) Using a graduated cylinder, measure 900 ml of acetonitrile and place it into a clean carboy with spigot.
 - (2) Add 100 ml deionized or distilled water.
 - (3) Label the container stating the mixture (90 percent acetonitrile and 10 percent water), date of preparation, and initials of technician who prepared the solution.
 - (4) Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 9 parts acetonitrile to 1 part of deionized or distilled water.

9.3 FLUOROMETER CALIBRATION

- a. Turn the power on and respond as indicated (no warm-up period is required).
- b. When first turned on, the fluorometer will go through a series of self-tests. After the self-tests are completed, the screen will allow you to change the date and time, or continue. You will then come to the "METHOD" screen. This screen allows you to choose a particular method, print a list of methods, or set up a new method. If the fluorometer has not yet been programmed with the appropriate method, use the following directions.
 - (1) Press the "Set Up" key.
 - (2) Enter the method number according to the sample matrix being tested and press "Enter".
 - (3) Enter the delay time as 40 seconds.

- (4) Select the measurement as "ppb".
 - (5) Select the results as "decimals".
 - (6) Enter the **High Calibrator** value according to the calibration card provided.
 - (7) Enter the **Low Calibrator** value as stated and continue with the procedure.
- c. Press the "Select" key.
 - d. Enter the method number according to the sample matrix being tested and press "Enter".
 - e. After choosing the method desired, place the high and low calibrator ampules in the fluorometer when specified.
 - f. For the first sample, insert the control into the fluorometer and read the result. The control range will read as stated on the control ampule bottle. If the reading is not in this range, try recalibrating the machine. If the control value is still out of range, contact the Technical Services Department at Romer Labs Inc. at 1-800-769-1380.

9.4 EXTRACTION PROCEDURES

- a. Transfer 50 grams of ground sample into an extraction mixing jar.
- b. Add 100 ml of the extraction solvent.
(methanol/ water for corn, corn meal, rice, popcorn, sorghum, and wheat)
(acetonitrile/water for soybeans and corn/soy blend)
- c. Cover the extraction jar and blend on high speed for 1 minute.
- d. Remove the cover and funnel the extract through a Whatman No.1 filter or a coffee filter into a sample jar labeled with the sample identification.
- e. After collecting the filtrate, remove the funnel, filter, and ground material and place over an empty collection container (e.g., disposable plastic beaker).

9.5 TEST PROCEDURES

a. Purification of Corn, Corn Meal, Rice, Popcorn, Sorghum, and Wheat.

- (1) Make sure the clear plastic tip is pushed firmly onto the bottom of the UniSep 2001 column. This prevents any solution from passing through the column prematurely.
- (2) Place 1 ml of the extract in the top of the column and discard the pipette tip.
- (3) Add 1 ml of the diluent and discard the pipette tip.
- (4) Place the blue cap on top of the column and mix thoroughly by hand, shaking vigorously for 5 seconds.
- (5) Uncap the top and bottom of the column and place the column in a 12 x 75 mm cuvette. Insert the syringe barrel and stopper into the top of the column.
- (6) Slowly (**30-40 seconds**) push the extract through the column until air comes out of the bottom.

NOTE: It is critical to push the solution completely through the column in at least a 30-40 second time-frame indicated to insure a complete extraction solution purification.

- (7) Transfer 0.5 ml of each purified sample extract to a clean 12 x 75 mm cuvette and cap.

b. Purification of Soybeans and Corn/Soy Blend.

- (1) Make sure the clear plastic tip is pushed firmly onto the bottom of the UniSep 2001 column. This prevents any solution from passing through the column prematurely.
- (2) Place 2 ml of the extract in the top of the column and discard the pipette tip.

- (3) Place the blue cap on top of the column and mix thoroughly by hand, shaking vigorously for 5 seconds.
- (4) Uncap the top and bottom of the column and place the column in a 12 x 75 mm cuvette. Insert the syringe barrel and stopper into the top of the column.
- (5) Slowly (**30-40 seconds**) push the extract through the column until air comes out of the bottom.

NOTE: It is critical to push the solution completely through the column in at least a 30-40 second time-frame indicated to insure a complete extraction solution purification.

- (6) Transfer 250 µl (0.25 ml) of each purified sample extract to a clean 12 x 75 mm cuvette.
- (7) Add 250 µl of methanol to the cuvette and cap.

c. Derivatization and Fluorometric Reading.

- (1) Immediately add 1 ml of the developer working reagent to each purified sample.
- (2) Recap the tube and vortex for 5 seconds.
- (3) Wipe the cuvette with lint-free paper and place in the fluorometer for a reading.
- (4) After a 40-second delay, the result will appear on the fluorometer screen and a record will be printed out.

NOTE: Once the developer reagent is added, the sample must be mixed, and the sample cuvette must be placed in the fluorometer quickly. Samples must be derivatized one sample at a time and then read before proceeding to the next sample.

9.6 REPORTING AND CERTIFYING TEST RESULTS

- a. Record the digital readout as ppb total aflatoxins in the sample.
- b. Report all results on the pan ticket and the inspection log to the nearest whole ppm.
- c. Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- d. Refer to the Certification section of the handbook for more detailed certification procedures.

9.7 SYSTEM CHECK

- a. Positive Control Option.

The test kit contains a positive control standard that may be used as a check on method technique and overall system performance.

- b. System Check Procedures.

- (1) Make sure the clear plastic tip is pushed firmly onto the bottom of the UniSep2001 column.
- (2) Place 1 ml of **80/20 methanol/water solution** in the top of the column and discard the pipette tip.

Note: Do not use the 90/10 acetonitrile/water solution in this procedure.

- (3) Add 1 ml of Positive Control Standard and discard the pipette tip.
- (4) Place the blue cap on top of the column and mix thoroughly by hand shaking vigorously for 5 seconds.
- (5) Uncap the top and bottom of the column and place the column in a 12 x 75 mm cuvette. Insert the syringe barrel and stopper into the top of the column.
- (6) Slowly (**30-40 seconds**) push the extract through the column until air comes out the bottom.

- (7) Transfer 0.5 ml of the purified positive control solution to a clean 12 x 75 mm cuvette and cap.
- (8) Immediately add 1 ml of developer and working reagent to the positive control standard.
- (9) Recap the tube and vortex for 5 seconds.
- (10) Wipe the cuvette with lint-free paper and place in the fluorometer for a reading.
- (11) After a 40-second delay, the result will appear on the fluorometer screen and a record will be printed out. The value received should fall within the range listed on the label of the bottle.

NOTE: Be sure to use the corn calibration factors to test the control standard.

9.8 SUPPLEMENTAL ANALYSIS

a. Diluting the Sample Extract.

If quantitative results are above the test method's conformance limit, test results are reported as exceeding the limit. To determine and report an aflatoxin level higher than 300 ppb, the sample extract must be diluted so that a value between 5 and 300 ppb is obtained. The final aflatoxin concentration is calculated by multiplying the results with the diluted extract by the dilution factor.

b. Example.

If the original analysis reported the aflatoxin value at 700 ppb, the sample extract would be diluted using the following procedures in order to obtain a true value.

- (1) Dilute 5 ml of the original extract with 10 ml of the extraction solvent mixture (**methanol/ water for corn, corn meal, rice, popcorn, sorghum, and wheat, acetonitrile/water for soybeans and corn/soy blend**). The total volume is 15 ml. This is a 1 to 3 dilution (compares volume in the beginning with the total volume in the end).

- (2) Multiply the analytical results obtained by 3 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the original value obtained with the diluted extract, the actual concentration in the original sample was 720 ppb.

$$\text{True Aflatoxin Value} = \frac{\text{Total Volume} \times \text{Aflatoxin Result}}{\text{Initial Extract Volume}}$$

$$\begin{aligned}\text{True Aflatoxin Value} &= (15 \div 5) \times 240 \text{ ppb} \\ &= 3 \times 240 \text{ ppb} = 720 \text{ ppb}\end{aligned}$$

9.9 CLEANING LABWARE

a. Negative Tests (≤ 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

9.10 WASTE DISPOSAL

a. Negative Results (\leq 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results ($>$ 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

9.11 EQUIPMENT AND SUPPLIES

- a. Blender with $\frac{1}{2}$ pint jars.
- b. Syringe with rubber stopper.
- c. Cuvette rack.
- e. Pipettor and tips - 200 to 1000 μl (.20 to 1 ml) adjustable.
- f. Vortex Mixer.
- g. Fluorometer (Vicam Series III or IV, or Romer RL100) and printer.
- h. 100 ml graduated cylinder.
- i. Funnel.
- j. Timer.
- k. Whatman No.1 Filter Paper or Coffee Filters.

- l. Glass cuvettes (12 x 75 mm).
- m. Empty bottles for Developer Working Reagent and Working Diluent.
- n. Repipette Dispenser (1ml), Labindustries Model LS830X3 or equivalent.
- o. Sample grinder.
- p. Balance.
- q. HPLC grade Methanol (**for extraction solvent for corn, corn meal, rice, popcorn, sorghum, and wheat**).
- r. Acetonitrile (**for extraction solvent for soybeans and corn/soy blend**).
- s. Deionized or Distilled Water.
- t. UniSep 2001 Fluoroquant "A" columns.
- u. Developer Concentrate.
- v. Diluent (**for corn, corn meal, rice, popcorn, sorghum, and wheat**).
- w. High, Low, and Control calibrator ampules.
- x. Positive Control Standard.

9.12 STORAGE CONDITIONS

- a. UniSep 2001 columns - Room temperature in a sealed container.
- b. Developer Concentrate - shipped in an amber bottle. Store in a tightly closed container in a cool, dry, well ventilated area away from direct sunlight, combustible materials, and incompatible substances.
- c. Calibrators - Room temperature.
- d. Diluent - In a cool place away from heat source.
- e. Positive Control Standard - Refrigerated at 23° to 32° F.

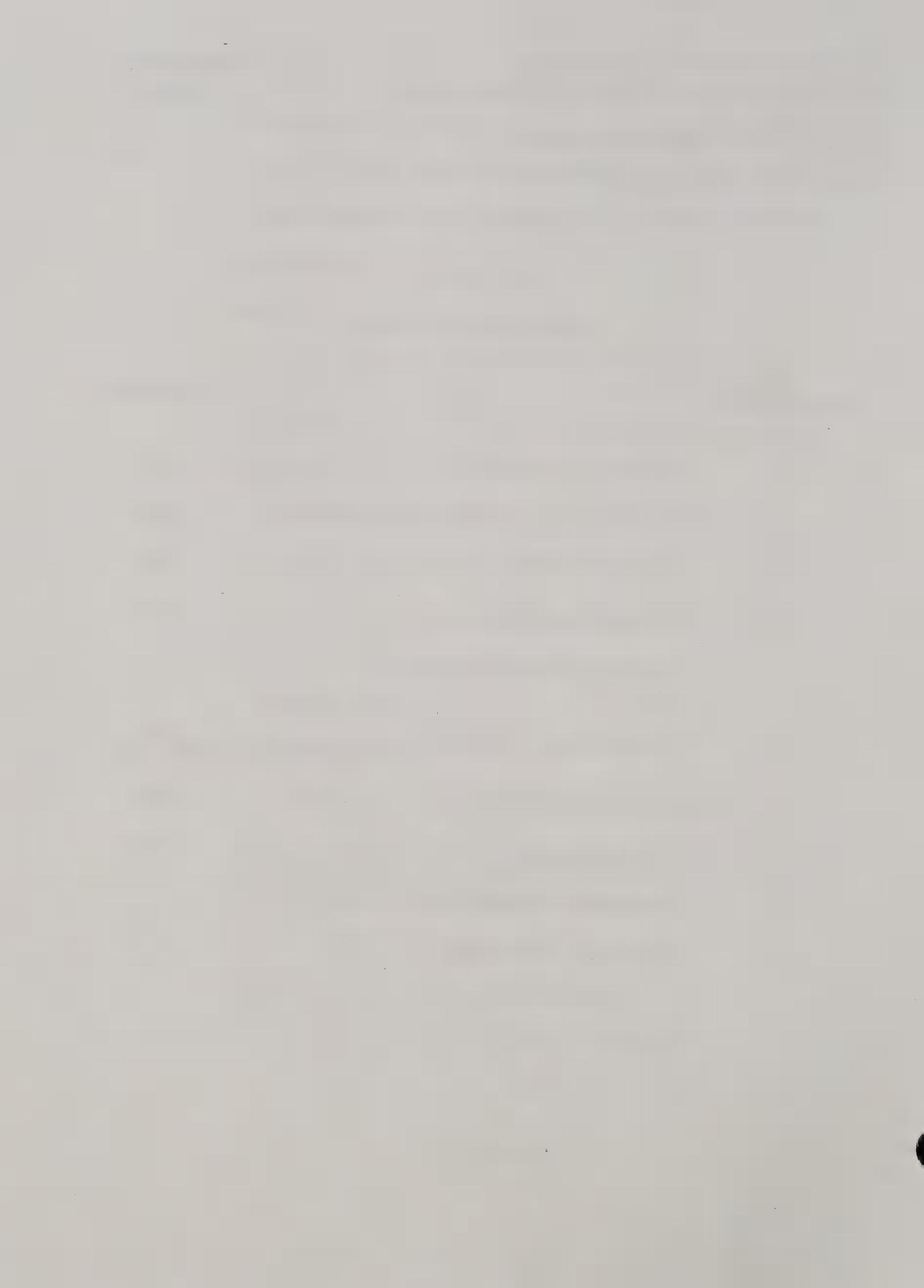
U.S. DEPARTMENT OF AGRICULTURE
GRAIN INSPECTION, PACKERS AND STOCKYARDS
ADMINISTRATION
FEDERAL GRAIN INSPECTION SERVICE
STOP 3630
WASHINGTON, DC 20090-3630

AFLATOXIN HANDBOOK
CHAPTER 10
3-4-02

CHAPTER 10

VERATOX-AST TEST KIT

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
10.1	GENERAL INFORMATION	10-1
10.2	PREPARATION OF EXTRACTION SOLUTION ...	10-1
10.3	EXTRACTION PROCEDURES	10-1
10.4	TEST PROCEDURES	10-2
10.5	REPORTING AND CERTIFYING TEST RESULTS.....	10-7
10.6	SUPPLEMENTAL ANALYSIS	10-7
10.7	CLEANING LABWARE	10-8
10.8	WASTE DISPOSAL.....	10-9
10.9	EQUIPMENT AND SUPPLIES.....	10-10
10.10	STORAGE CONDITIONS.....	10-11



10.1 GENERAL INFORMATION

The Veratox AST test is a quick diagnostic tool to predict the presence of aflatoxin in corn and other commodities. The kit uses an enzyme-linked immunosorbent assay (ELISA) technique to obtain quantitative results from absorbance readings at 650 nm when sample readings are compared to a 20 ppb control and a pre-generated standard curve (0 to 400 ppb).

10.2 PREPARATION OF EXTRACTION SOLUTION

The extraction solvent used in the Veratox test method is a methanol/water (distilled or deionized) mixture consisting of 70 percent methanol (ACS grade or better) and 30 percent water.

- a. Using a graduated cylinder, measure 700 ml of methanol and place it into a clean carboy with spigot.
- b. Add 300 ml deionized or distilled water to the methanol and shake vigorously until it is completely mixed.
- c. Label the container stating the mixture (70 percent methanol and 30 percent water), date of preparation, and initials of technician who prepared the solution.
- d. Store this solution at room temperature in a tightly closed container until needed.

NOTE: To prepare smaller or larger amounts of solution use the ratio of 7 parts methanol to 3 parts of deionized or distilled water.

10.3 EXTRACTION PROCEDURES

- a. Place a sheet of filter paper (Whatman 2V folded or S&S 24 cm pleated or equivalent) into a clean funnel mounted over a 25 X 200 mm (diameter x length) test tube or a collection beaker.
- b. Label the collection container with the sample identification.
- c. Place the 50-gram portion of the ground sample into the blender container.

- d. Pour in 250 ml of the 70/30 percent methanol/water solution and securely close the blender top.
- e. Blend for exactly two minutes at high speed.
- f. Pour the resultant mixture from the blender into the funnel containing the filter paper and collect approximately 25 ml of extract.
- g. Filter approximately 50 ml of water through the filter containing the ground material and allow to drain.

10.4 TEST PROCEDURES

a. Preparation of Solutions.

- (1) Place 3 ml of substrate (light green labeled bottle) solution into a clean, labeled reagent boat. Cover boat to protect solution from dust and light.

NOTE: Do not return any substrate solution to the original bottle once it has been removed.

- (2) Place 3 ml of Red Stop (red labeled bottle) solution into a clean reagent boat. Cover boat to protect solution from dust and light.

b. Sample Analysis.

Do not use reagents or microwells from one kit serial number with reagents/wells from a different serial number. Reagent boats may be rinsed and reused.

- (1) Open foil bag and remove 3 red-marked mixing wells for each sample to be tested (maximum of 4 samples or 12 wells). Place them in the microwell holder, and mark the left end of each strip with a "1."

Do not run more than four samples at one time.

- (2) Remove 3 antibody wells for each sample to be tested (maximum of 4 samples or 12 wells). Place them in the microwell holder, and mark the left end of each strip with a "1."
- (3) Reseal bag by folding over and tightly closing with a suitable fastener (large paper clip, tape, or suitable dust and light protectant).

- (4) Place 100 µl of conjugate (blue-labeled bottle) into each mixing well using a 100 µl pipettor with a new tip. Prime the pipette tip first before dispensing the 100 µl. Discard the pipette tip.

NOTE: "Prime the pipette tip" is accomplished by drawing liquid up into the tip and dispensing it back into the bottle once or twice.

- (5) Place 100 µl of control (yellow-labeled bottle) into the first mixing well labeled "1." Prime the tip before dispensing. If testing more than one sample, also place 100 µl of control into mixing well #4 for the second sample, mixing well #7 for the third sample, and mixing well #10 for the fourth sample. Discard the pipette tip.
- (6) Place 100 µl of sample each in mixing wells #2 and #3. Prime the tip first before dispensing. Discard the tip. Subsequent samples should be placed in wells #5 and #6, then #8 and #9, and then #11 and #12.

See the diagram below for an example of the procedure.

	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
mixing wells	O	O	O	O	O	O	O	O	O	O	O	O
	C	S1	C	S2	C	S3	C	S4	C	S5	C	S6

"W" = well number (e.g., #1 through #12)

"C" = control

"S1, S2, S3, & S4" = sample numbers

- (7) Using the 12 channel pipettor and the overfill method (see note below), mix the contents of the mixing wells by pipetting up and down in the tips 5 times.

NOTE: The "overfill method" is performed by drawing greater than 100 µl into the pipette tips by pressing the pipettor to the second stop before placing tips into the solution. Place tips into the liquid and release the plunger slowly and completely.

- (8) Transfer 100 µl to the antibody coated wells (the unmarked, clear wells). To dispense only 100 µl, press plunger to the first stop.
- (9) Mix in the antibody coated wells by gently sliding the microwell holder back and forth on a horizontal surface for 15 seconds. Be careful not to allow solution to splash out of wells.
- (10) Immediately following mixing, incubate for 5 minutes. Discard all mixing (red marked) wells and tips.
- (11) With a wash bottle containing deionized/distilled water, fill each antibody well and dump the contents into a waste receptacle. Repeat this step five times.
- (12) Turn microwell holder, with wells in it, upside down on a paper towel and tap gently until water is removed from the wells.
- (13) Using the 12 channel pipettor and the overfill method, place 100 µl of substrate into each well.
- (14) Mix gently by sliding the microwell holder back and forth for 15 seconds on a horizontal surface for 15 seconds. Be careful not to allow solution to splash out of wells.
- (15) Immediately following mixing, incubate for 5 minutes.
- (16) Discharge the remaining substrate in the pipette tips by plunging once or twice without drawing any additional liquid up into the tips. Save these tips for the next step.
- (17) Using the 12 channel pipettor and the overfill method, add 100 µl of the red stop solution (red labeled bottle) into each well.
- (18) Mix gently by sliding the microwell holder back and forth for 15 seconds. Again be careful not to lose any solution from the wells. Visually check the appearance of the wells. Discard all pipette tips.
- (19) Read in a microwell reader using a 650 nm filter within 5 minutes of the addition of the red stop solution.

c. Reading Results with Microwell Reader.

- (1) Turn on the power to reader at the beginning of the test procedure to allow the electronics to stabilize. Make sure that the reader is properly attached to the computer.
- (2) Turn on the computer and insert the VERATOX-AST software disc into the drive slot.
- (3) Start the VERATOX program and select option "A" - "RUN AST."
- (4) Check the kit identification and the standard curve values with the Standard Curve Program Calculated Points that came with the test kit. Edit standard or kit lot numbers as necessary.
- (5) Press the "Enter" key, then press the "R" key to ready the computer to receive data from the microwell reader.
- (6) Calibrate the microwell reader by following the instructions which appear on the LCD window of the reader.
 - (a) Remove sample carrier and press the "Enter" key.
 - (b) Place the filter holder in the W2 position and press "Enter." The instrument will calibrate on the W2 filter.
 - (c) Move the filter holder to the W1 position and press "Enter." The instrument will calibrate on the W1 filter.

NOTE: The Micro-well reader used in the official aflatoxin testing service is designed to do several testing functions. Each function requires specific set-up parameters. The required parameters for aflatoxin testing are: "F1 set up L S P, 12S, ABSORB, N Y N." To ensure that the Micro-well reader is properly set for aflatoxin testing, periodically check the display set-up as follows:

- (d) Press the Display Set up. Display should read F1 setup L S P 12S ABSORB N Y N.

If the display reads differently, contact the Neogen Corporation representative for instruction. Otherwise, press the Display Set up again. This will return the instrument to normal operational mode.

- (e) Press the "Clear" key, then the "Blank" key. This will blank the instrument on air and it is now ready to measure absorbance.
- (f) Place the wells into the reader's sample holder. Make sure that the well marked "1" is in the far left position in the holder.
- (g) Move the holder to the left so that the first well is under the reader and press the "Read" key. Repeat this process until all wells are read.
- (h) Follow the instructions as requested by the software. Values displayed on the computer screen will be the mean of the duplicate measurements.

d. Troubleshooting.

The Veratox AST quantitative test requires two duplicate portions of a sample to be run in addition to a control portion. The mean OD reading of the duplicate portions is compared with the calculated OD points in the standard curve program to obtain a result in ppb. If the coefficient of variance (CV) between the OD values of the test portions is not within the required 15 percent range of difference, a "CV overrange" error will be displayed as the test result. Typically, a "CV overrange" message indicates that the test results are not valid and that another portion must be tested.

If the OD readings of the duplicate portions both indicate that the aflatoxin level is less than 300 ppb and a "CV overrange" message appears, then an additional sample portion must be tested until the OD values of duplicate portions are within 15 percent of the mean OD and the "CV overrange" message is eliminated. If the OD readings for the duplicate portions indicate that one of the portions exceeds 300 ppb and the other portion is less than 300 ppb and a "CV overrange" message is displayed, the sample must be retested (full strength or diluted portion) until both of the OD readings are within the 15 percent range.

If the OD readings for the duplicate portions both indicate that the aflatoxin content exceeds 300 ppb but a "CV overrange" message appears, then:

- (1) an additional sample portion (diluted) must be tested until the OD values of duplicate portions are within 15 percent of the mean OD,
- or
- (2) official personnel may stop the testing if the applicant requests only a certification statement that aflatoxin exceeds 300 ppb. In this instance, official personnel will certificate the aflatoxin testing results as a "Aflatoxin exceeds 300 ppb."

10.5 REPORTING AND CERTIFYING TEST RESULTS

- a. Report all results on the pan ticket and the inspection log to the nearest whole ppm.
- b. Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- c. Refer to the Certification section of the handbook for more detailed certification procedures.

10.6 SUPPLEMENTAL ANALYSIS

- a. Diluting the Sample Extract.

If quantitative results are above the test method's conformance limit, test results are reported as exceeding the limit. To determine and report an aflatoxin level higher than 300 ppb, the sample extract must be diluted so that a value between 5 and 300 ppb is obtained.

The final aflatoxin concentration is calculated by multiplying the results with the diluted extract by the dilution factor.

b. Example.

If the original analysis reported the aflatoxin value at 700 ppb, the sample extract would be diluted using the following procedures in order to obtain a true value.

- (1) Dilute 5 ml of the original extract with 10 ml of the extraction solvent mixture. The total volume is 15 ml. This is a 1 to 3 dilution (compares volume in the beginning with the total volume in the end).
- (2) Multiply the analytical results obtained by 3 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the original value obtained with the diluted extract, the actual concentration in the original sample was 720 ppb.

$$\text{True Aflatoxin Value} = \frac{\text{Total Volume} \times \text{Aflatoxin Result}}{\text{Initial Extract Volume}}$$

$$\begin{aligned}\text{True Aflatoxin Value} &= (15 \div 5) \times 240 \text{ ppb} \\ &= 3 \times 240 \text{ ppb} = 720 \text{ ppb}\end{aligned}$$

10.7 CLEANING LABWARE

a. Negative Tests (≤ 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution". Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

10.8 WASTE DISPOSAL

a. Negative Results (\leq 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

10.9 EQUIPMENT AND SUPPLIES

a. Materials Supplied in Test Kits

- (1) 48 antibody-coated wells.
- (2) 48 red-marked mixing wells.
- (3) 1 yellow-labeled bottle of 1.5 ml 20 ppb aflatoxin control.
- (4) 1 blue-labeled bottle of 7 ml aflatoxin-HRP conjugate solution.
- (5) 1 green-labeled bottle of 24 ml K-blue substrate solution.
- (6) 1 red-labeled bottle of 32ml red stop solution.

b. Materials Required but not Provided:

- (1) Methanol - ACS grade or better.
- (2) Deionized or distilled water.
- (3) 250 ml graduated cylinder.
- (4) Whatman 2V folded or S&S 24 cm pleated (or equivalent) filter paper.
- (5) Filter funnel.
- (6) Sample collection tubes.
- (7) Blender with mixing jars.
- (8) Balance.
- (9) Sample grinder.
- (10) EL 301 Microwell strip reader with 650 nm filter.
- (11) 12-channel pipettor.

- (12) 100 µl pipet.
- (13) Pipette tips.
- (14) Microwell holder.
- (15) Waterproof marker.
- (16) 2 reagent boats (to hold substrate and red stop solutions).
- (17) Timer.

10.10 STORAGE CONDITIONS

- a. The kit is packaged in a sealed "foil bag" with a label indicating the lot number and expiration date.
- b. Store test kits between 36°- 46°F when not in use. Avoid prolonged storage of kits at room temperature. Do not freeze test kits.
- c. Bring kits up to room temperature 64°- 86°F prior to use.
- d. Do not use kit components beyond their expiration date.

NATIONAL AGRICULTURAL LIBRARY



1022468742

